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(54) Title: INFLUENZA VACCINE COMPOSITIONS CONTAINING 3-O-DEACYLATED MONOPHOSPHORYL LIPID A

(57) Abstract

The present invention provides vaccine compositions capable of enhancing a protective response to a selected influenza antigen, said composition containing at least the antigen and 3D-MPL, and methods of enhancing an immune response to influenza using these compositions.

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INFLUENZA VACCINE COMPOSITIONS CONTAINING 3-O-DEACYLATED MONOPHOSPHORYL LIPID A

Cross-Reference to Other Applications

This is a continuation-in-part application of co-pending United States patent application serial number 021,535, filed February 19, 1993.

Field of the Invention

This invention relates to vaccines useful in preventing infection with influenza in humans.

Background of the Invention

Influenza virus infection causes acute respiratory disease in man, horses and fowl, sometimes of pandemic proportions. Influenza viruses belong to the orthomyxovirus family of RNA viruses and, as such, have enveloped virions of 80 to 120 nanometers in diameter, with two external glycoprotein spikes, hemagglutinin (HA) and neuraminidase (NA), and five internal proteins, nucleoprotein, matrix protein and three polymerases. Influenza viral RNA also codes for two non-structural proteins (NS1 and NS2) which are produced in infected cells, but are not incorporated into infectious virions.

Three types of influenza virus, Type A, Type B and Type C, infect humans. Type A viruses have been responsible for the majority of human epidemics in modern history, although there are also sporadic outbreaks of Type B infections. Known swine, equine and fowl viruses have mostly been Type A, although Type C viruses have also been isolated from swine.

Within a virus, genetic variation in the surface proteins HA and NA has resulted in three important subtypes, designated H1N1, H2N2 and H3N2. Within Type A, subtypes H1 ("swine flu"), H2 ("asian flu") and H3 ("Hong Kong flu") are predominant in human infections.

Influenza viruses continually undergo genetic change in their surface glycoproteins which affects antigenic variation. This is most pronounced within the A virus type, where major genetic changes in the HA or NA

proteins have already occurred ("antigenic shifts"). The emergence of these new virus subtypes have caused a pandemic spread of infection resulting in significant mortality and morbidity. For example, the H1N1 viruses, prevalent before 1957, were replaced by the H2N2 virus subtype which remained predominant until 1968, when they were, in turn, replaced by the H3N2 subtype. Currently, H3N2 strains are still circulating, but since 1977, H1N1 viruses have re-emerged. The HAs within a given subtype also undergo smaller genetic changes (point mutations) every year or two ("antigenic drift"). These are largely restricted to antigenic determinants clustered around the sialic acid binding site in the HA1 and result in the emergence of new virus strains. Although this antigenic drift does not cause serious mortality and morbidity to the extent caused by antigenic shift, it is responsible for yearly influenza epidemics.

Influenza vaccines are classified into three types, whole-virion, split, and subunit. Whole-virion vaccines, based on intact viral particles, although generally more immunogenic, tend to be more reactogenic and are therefore being replaced by split and subunit vaccines which are prepared from purified viral components obtained after disruption of the virus by treatment with various chemical agents. The distinction between split and subunit vaccines resides in the fact that subunit vaccine contain almost exclusively haemagglutinin and neuraminidase, the surface antigens of the virus, whereas split vaccines contain in addition variable amounts of internal components of the virus such as the ribonucleoprotein and the matrix protein.

Currently available commercial influenza vaccines are based on the principle that antibody to HA or NA confers protection. They consist of non-adjuvanted, inactivated, whole, or split virus products utilizing virus grown in embryonated hen's eggs. All influenza vaccines currently contain preparations from H1N1, H3N2, and type B virus strains. Due to the annual antigenic variation, specific virus strains are updated on a yearly

basis according to WHO recommendations, which are based on epidemiological surveillance of prelevent circulating virus strains.

There is no "universal" influenza virus vaccine, i.e., a non-strain specific vaccine. Recently, attempts have been made to prepare such universal, or semi-universal, vaccines from reassortant viruses prepared by crossing different strains. More recently, such attempts have involved recombinant DNA techniques focusing primarily on the HA protein.

Influenza vaccines are under utilized for a variety of reasons including doubts about efficacy, fear of side effects, need for annual revaccination, and lack of interest among providers. Present vaccines have demonstrated efficacy ranging from approximately 60-80% against infection with influenza viruses that are antigenically closely related to the virus strains used in the vaccine. This rate of protective efficacy tends to decrease when the HA antigen of the epidemic strain HA "drifted" away from the vaccine strain and would fall to zero if a "shift" in subtype occurs. In addition, protection appears to be diminished in some immunocompromised groups, such as the elderly living in nursing homes.

Thus the major drawbacks of currently available vaccines result from the fact that frequent antigenic drift dictates that the component virus strains be changed annually and individuals must undergo revaccination.

There remains a need in the art for vaccine formulations and compositions capable of inducing protective responses in animals for a wide variety of pathogens.

Summary of the Invention

In one aspect, the present invention provides a vaccine composition capable of stimulating an enhanced immune and protective response in a vaccinated animal against influenza, the composition comprising a selected

influenza antigen or antigenic polypeptide and an effective amount of 3-o-deacylated monophosphoryl lipid A (3D-MPL).

In another aspect, the invention provides a vaccine composition comprising a selected influenza antigen or antigenic polypeptide, an effective amount of 3D-MPL and a liposome preparation. The liposome preparation is defined herein and, in addition to acting as a carrier, acts as an adjuvant and offers significant manufacturing and formulation advantages.

In a further aspect, the invention provides a method for enhancing a vaccinee's immune response to a selected influenza antigen. This method involves administering to a mammal, preferably a human, a vaccine composition described above.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a bar graph illustrating cross-protection for H1N1 and H2N2 subtype influenza viruses in mice immunized with Flu D protein (SK&F 106160) in aluminum plus 3D-MPL, as described in Example 18.

Fig. 2A is a bar graph illustrating splenic proliferative responses pre-challenge in mice vaccinated with flu D formulations and controls. See Example 20.

Fig. 2B is a bar graph illustrating splenic proliferative responses post-challenge in mice vaccinated with flu D formulations and controls. See Example 20.

Fig. 3A is a bar graph illustrating lymph node proliferative responses obtained on day 4 in mice vaccinated with 20 µg flu D in aluminum (open bars) or aluminum and 3D-MPL (cross-hatched bars). See Example 21.

Fig. 3B is a bar graph illustrating lymph node proliferative responses obtained on day 4 in mice vaccinated with 5 µg flu D in aluminum (open bars) or aluminum and 3D-MPL (cross-hatched bars). See Example

21.

Fig. 3C is a bar graph illustrating lymph node proliferative responses obtained on day 4 in mice vaccinated with 1 µg flu D in aluminum (open bars) or aluminum and 3D-MPL (cross-hatched bars). See Example 21.

Fig. 4A is a bar graph illustrating proliferation on day 2 by immune lymph nodes in mice immunized with 1 µg D protein vaccine formulation. See Example 21.

Fig. 4B is a bar graph illustrating proliferation on day 3 by immune lymph nodes in mice immunized with 1 µg D protein vaccine formulation. See Example 21.

Fig. 4C is a bar graph illustrating proliferation on day 4 by immune lymph nodes in mice immunized with 1 µg D protein vaccine formulation. See Example 21.

Fig. 4D is a bar graph illustrating IL-2 production on day 2 by immune lymph nodes in mice immunized with 1 µg D protein vaccine formulation. See Example 21.

Fig. 4E is a bar graph illustrating IL-2 production on day 3 by immune lymph nodes in mice immunized with 1 µg D protein vaccine formulation. See Example 21.

Fig. 4F is a bar graph illustrating IL-2 production on day 4 by immune lymph nodes in mice immunized with 1 µg D protein vaccine formulation. See Example 21.

Fig. 5A is a graph demonstrating interferon levels in antigen-stimulated cultures from mice immunized as described in Example 24 below.

Fig. 5B is a graph demonstrating IL-2 levels in antigen-stimulated cultures obtained from mice immunized as described in Example 24 below.

Fig. 6A is graph showing virus titers determined in the nose by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group) for a control containing alum and 3D-MPL (--square--), influenza monovalent split vaccine containing A/PR/8 strain with no adjuvant (--triangle--), and a strain A/PR/8 adjuvanted with 3D-MPL (solid line and circle). See Example 28.

Fig. 6B is graph showing virus titers determined in the nose by MDCK microassay on days 1, 3, 5, 7 and 9

post-challenge (5 mice per group) for a control containing alum and 3D-MPL (--square--), influenza monovalent split vaccine containing Singapore strain with no adjuvant (--triangle--), and the Singapore strain adjuvanted with 3D-MPL (solid line and circle). See Example 28.

Fig. 6C is graph showing virus titers determined in the trachea by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group) for the same three vaccine formulations as in Fig. 6A.

Fig. 6D is graph showing virus titers determined in the trachea by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group) for the same three vaccine formulations as in Fig. 6B.

Fig. 6E is graph showing virus titers determined in the lung by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group) for the same three vaccine formulations as in Fig. 6A.

Fig. 6F is graph showing virus titers determined in the lung by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group) for the same three vaccine formulations as in Fig. 6B.

Detailed Description of the Invention

The present invention provides vaccine compositions capable of eliciting an enhanced immune response in vaccinated hosts, including humans, as well as methods for preparing and using such vaccine compositions. A vaccine composition of this invention is characterized by containing an effective amount of a selected influenza antigen or antigenic polypeptide and 3-o-deacylated monophosphoryl lipid A (3D-MPL). Optionally, a liposome preparation may also be a component of the vaccine compositions of this invention.

The inventors have discovered that the combination of 3D-MPL and certain influenza antigens are effective in achieving protective responses against influenza, which are not achieved by the influenza antigen alone. For example, with the antigenic polypeptide known as Flu D,

described below, this response is such that a lower amount of antigen is required to obtain the same results as are achieved with purified Flu D and Complete Freunds Adjuvant (CFA), a known strong adjuvant which is toxic to animals.

Further, when the selected influenza antigen and 3D-MPL are entrapped within a liposome as described herein, a protective response which exceeds that achieved by the antigen and any other adjuvant combination is obtained.

By the term "enhanced immune response" as used herein, is meant that the vaccinated host produces a stronger cellular immune response (protective T lymphocyte production) to the vaccine composition of the invention than is or would be produced by the host in response to the selected antigen when not adjuvanted, or when adjuvanted with other conventional adjuvants suitable for internal administration. An increased antibody (B cell) response is also anticipated by this enhanced response.

By the term "immunologically effective amount" or "effective amount" as used herein is meant that amount of antigen which induces a protective immune response.

By the terms "selected antigen", "antigenic polypeptide or protein" or "immunogen" as used herein is meant a whole or inactivated pathogen, an immunogenic protein, peptide or fragment from the pathogen, which is optionally fused to another peptide or protein which is of homologous or heterologous origin. These terms also include a split virus, defined below. These terms may also include non-proteinaceous biological materials from the pathogen. The pathogens are preferably disease-causing organisms which infect humans, although animal pathogens may also be employed in these vaccines, where desired for veterinary purposes. These terms refer to the ability of the whole pathogen, split virus, peptide or fusion protein to elicit a protective immune response in a vaccinated host.

By the term "monovalent vaccine" is meant a vaccine containing antigens from a single type or subtype of

influenza virus, e.g., H1N1, H2N2, H3N2 of Type A, Type B and Type C.

By the term "multivalent vaccine" is meant a vaccine containing antigens from more than a single type or subtype of influenza virus, i.e., a trivalent vaccine may contain antigens from any three influenza types or subtypes, e.g., H1N1, H2N2, H3N2 of Type A, Type B and Type C.

By the term "split virus" is meant an influenza virus suspension, obtained from embryonated hens' eggs inoculated with seed lot material, which is partially purified and concentrated. The concentrated virus suspension is treated with a detergent, such as sodium-desoxylcholate, to disrupt the virus particles. Removal of viral phospholipids during this splitting process produces an inactivated influenza antigen for which the reactogenicity potential is greatly reduced. The split virus suspension is completely inactivated by the combined effect of detergent and formaldehyde.

The following disclosure of the compositions and methods of this invention specifically describes vaccine compositions for prophylactic use against influenza virus.

In one preferred embodiment of this invention, a vaccine composition capable of eliciting an enhanced immune response protective against infection with influenza virus contains at least a selected influenza antigenic polypeptide, such as $NS1_{1-81}HA2_{65-222}$ (referred to herein as Flu D, the D protein or the Flu D protein), adjuvanted with 3D-MPL.

Currently, flu D protein is one preferred influenza antigenic polypeptide for use in the vaccine compositions of this invention because it is the most easily purified of the influenza fusion proteins which contain the entire carboxy-terminal region of HA2 portion of the hemagglutinin region. D protein comprises the first 81 amino acids of NS1 fused to amino acid 65 of the truncated HA2 subunit (amino acids 65-222). Optionally, as is the case with the other NS1-HA2 fusions proteins

disclosed herein, a linker sequence may be inserted between the two fused sequences.

In a presently preferred embodiment, the DNA coding sequence for flu D protein is prepared by as described in EP 0366238 by restricting the HA2 coding sequence with PvuII and ligating the C-terminal region of the NcoI site between amino acids 81 and 82 in the NS1 coding sequence via a synthetic oligonucleotide linker. This linker sequence codes for glutamine-isoleucine-proline. D protein, for which a 90-95% purity has been achieved, requires application of the purification methods described herein to substantially remove the host cell (*E. coli*) proteins and other contaminants.

The flu D protein and the recombinant expression and purification thereof are disclosed in detail in co-pending U. S. Patent Application SN 07/751,899 and in its corresponding European Patent Application No. 366,238, published May 2, 1990 and U. S. Patent Application Sn 07/387,558 and in European Patent Application No. 366,239, published May 2, 1990. These applications are incorporated by reference for the purpose of describing this protein, its expression and purification.

Other suitable influenza antigenic polypeptides in addition to flu D, may be used in the vaccine compositions of this invention including those described in European Patent Applications 366,238 and EP 366,239, both published May 2, 1990 and in co-pending U. S. Patent Applications SN 07/751,898, 07/751,896 and 07/837,773. Such proteins include ΔM, ΔM+, A, C, C13, C13 short, and ΔD. Other suitable include Cys-less D, $HA2_{66-222}$, and NS1H3HA2 constructs, such as those described in co-pending U.S. Patent Applications Ser. No. 07/751,898, 07/751,896, and 07/837,773 and WO 93/15763 incorporated by reference herein. Particularly desirable are the H3HA2 constructs referenced above. Recent studies by the inventors have shown that mice immunized with a "cocktail" containing both NS1-H1HA2 and NS1-H3HA2 fusion proteins were protected from lethal challenge with both H1 and H3-virus subtypes.

Coding sequences for the HA2, NS1 and other viral proteins of influenza virus can be prepared synthetically or can be derived from viral RNA by known techniques, or from available cDNA-containing plasmids as described in the above-incorporated published European applications. For example, in addition to the above-cited references, a DNA coding sequence for HA from the A/Japan/305/57 strain was cloned, sequenced and reported by Gething et al, Nature, 287:301-306 (1980); an HA coding sequence for strain A/NT/60/68 was cloned as reported by Sleigh et al, and by Both et al, both in Developments in Cell Biology, Elsevier Science Publishing Co., pages 69-79 and 81-89, (1980); an HA coding sequence for strain A/WSN/33 was cloned as reported by Davis et al, Gene, 10:205-218 (1980); and by Hiti et al, Virology, 111:113-124 (1981). An HA coding sequence for fowl plague virus was cloned as reported by Porter et al and by Emtage et al, both in Developments in Cell Biology, cited above, at pages 39-49 and 157-168.

Systems for cloning and expressing the vaccinal polypeptide in various microorganisms and cells, including, for example, *E. coli*, *Bacillus*, *Streptomyces*, *Saccharomyces*, mammalian and insect cells, are known and available from private and public laboratories and depositories and from commercial vendors.

The D protein can be purified by the process described below in Example 13. Various conventional procedures can be employed in connection with the purification of the proteins of the compositions of the present invention, although such other procedures are not necessary to achieve a highly purified, pharmaceutical grade product. Such procedures can be employed between, before or after the above described process steps. One such optional step is diafiltration, a form of continuous dialysis which is extremely effective in achieving many buffer exchanges. Diafiltration is preferably carried out across a cellulosic membrane or ultrafilter. Suitable membranes/filters are those having from about a 1000 molecular weight (MW) cut-off to those having pore

size up to 2.4 μm diameter. A number of different systems adaptable to diafiltration are commercially available, such as the 10K Amicon dual spiral cartridge system. In the process of the present invention, diafiltration using a 20 mM Tris buffer at about pH 8 can be effectively employed in the purification and subsequent concentration of the polypeptide to be employed in the vaccine composition of this invention.

In another preferred embodiment, the antigen used in a vaccine composition of the invention is a whole inactivated pathogen, such as a split virus, described in detail in Example 25. A monovalent split influenza vaccine containing a split virus or a multivalent (e.g. trivalent) split influenza vaccine containing more than one split virus, may also be adjuvanted with 3D-MPL. In one formulation, the vaccine contains the split virus prepared from an H1N1 strain, such as Singapore/6/86 [Sachsisches Serumwerkl GmbH (SSW), Dresden, German and the National Institute for Biological Standards and Control (NIBSC, London, England)] which is currently used in conventional flu vaccines. Alternatively, other H1N1 split viruses may be prepared from A/PR/8/34 (also called A/PR/8) described in T. Francis, Proc. Soc. Exp. Biol. Med., 32:1172 (1935) and available from the American Type Culture Collection, Rockville, Maryland, USA under ATCC No. VR-95. The monovalent vaccine contains antigens from one strain of influenza type virus. Alternatively, the vaccine may be multivalent, containing more than one influenza antigen, e.g., two or three split viruses, to increase the reactivity against more than one influenza type virus. An example of a trivalent vaccine includes, for example, the H1N1 Singapore/6/86 strain, with an H3N2 strain Beijing/32/92 [SSW, Dresden, Germany], and a Type B strain, Panama/45/90 [SSW, Dresden, Germany].

Influenza viruses, which can be prepared as split viruses by known means as described in Example 25 include any strains, subtypes and types, particularly those recommended by WHO, many of which are available from clinical specimens and from public depositories, such as

the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, USA (ATCC) and NIBSC. For example, other suitable H3N2 virus strains include, without limitation, A/Victoria/H3/75 described in Fiers et al, Cell, 19: 683-696 (1980); A/Udorn described in C. J. Lai et al, Proc. Natl. Acad. Sci., USA, 77:210-214 (1980); Palese and Schulman, Virol., 57:227-237 (1974); and A/HK/8/68, described in WHO Weekly Epid. Record, vol. 43:401 (1968) and available from the ATCC as No. VR-544 as well as Beijing/32/92. Other suitable Type B strains include, without limitation, those known as B/Lee/40 described by Krystal et al, Proc. Natl. Acad. Sci., USA, 79:4804-4900 (1982) and B/Taiwan, available from the ATCC as No. VR-295, Panama/45/90, and B/Yamaghta strains. H2N2 viruses may also be useful in these vaccines.

It should be understood that in addition to other influenza viruses or inactivated viral preparation, other antigenic materials from other pathogens are anticipated to be employed in addition to the exemplified antigens following the teachings contained herein.

Another component of a vaccine composition of this invention is the adjuvant, 3D-MPL. 3D-MPL is described in detail in U. S. Patent No. 4,912,094, incorporated by reference herein, and is commercially available from RIBI Immunochem Research Inc., Hamilton, Montana. Briefly, 3D-MPL is a derivative of the endotoxin, mono-phosphoryl Lipid A (MPL), a Lipid A analog isolated from a heptoseless, RE mutant of a Gram-negative bacteria, such as *Salmonella minnesota*. MPL lacks a phosphate group at the C-1 position of glucosamine. Treatment of the MPL molecule to remove the acyl chain at position 3 of glucosamine, yields 3D-MPL. 3D-MPL is non-toxic, in contrast to other enterobacterial lipopolysaccharides, but retains the antigenic activity of the parent endotoxin. This molecule is useful in preventing Gram-negative sepsis and endotoxemia.

3D-MPL can be dissolved in water to yield solutions of vesicular aggregates, which are presumably composed of lipid bilayer membranes. Thus, it is not likely that 3D-

MPL is seen as individual molecules by the immune system, but rather interacts by membrane-membrane contacts, involving cell surfaces and vesicle surfaces.

In a preferred embodiment, the particle size of the MPL is 'small' and in general does not exceed 120nm.

To make 3 deacylated monophosphoryl lipid A with a small particle size, in general not exceeding 120nm the procedure described in GB 2 220 211 may be followed (or commercial MPL of larger particle size may be purchased from Ribi Immunochem.) and the product may then be sonicated until the suspension is clear. The size of the particles may be estimated using dynamic light scattering as described hereinbelow.

Preferably the size of the particles is in the range 60-120nm.

Most advantageously the particle size is below 100nm.

According to the present invention, the inventors have determined that an influenza antigenic polypeptide, e.g., flu D protein, when highly purified, is neither immunogenic nor protective in the absence of adjuvant. However, when adjuvanted with 3D-MPL, as described herein and illustrated in Examples 14-24 below, the protein is capable of inducing protection against influenza infection. When the selected antigen is a split virus in a monovalent or trivalent composition, a composition of the present invention demonstrates an increase in immunogenicity and cross-reactivity.

Further, the inventors have discovered that when an influenza antigenic polypeptide, for example, flu D protein, is adjuvanted with 3D-MPL, a lower dose of flu D protein is required to achieve the same level of immune response obtained when the protein is adjuvanted with an aluminum adjuvant only. When the selected antigen is a split influenza virus in a monovalent or trivalent composition, it is anticipated that reactogenicity of the composition will be decreased by the use of less antigen in this embodiment of the invention.

Additional adjuvants may also be included in the

vaccine compositions of the invention. One desirable additional adjuvant is alum, or aluminum hydroxide or aluminum phosphate. When flu D protein is adjuvanted with a combination of aluminum and 3D-MPL, a level of potency is reached equivalent to that seen with Complete Freunds Adjuvant (CFA), the classic adjuvant for supporting T cell responses but which is not suitable for internal administration in humans.

To prepare a preferred vaccine composition of this invention containing an antigenic polypeptide, e.g., flu D protein and 3D-MPL, a desired amount of the flu D protein in admixed with a suitable amount of the 3D-MPL, as described in more detail below. Optionally, lyophilized lipid A is admixed with the pre-liposome gel described in detail below prior to the antigen. Most preferably, a molar ratio of phosphatides to lipid A is 66:1. However, the density of the agent may be varied to the desired level.

Other suitable agents for addition to the vaccine composition include, for example, IL-2, QS21 [C. R. Kensil et al, J. Immunol., 146(2):431-437 (1991)], and muramyl dipeptides (MDP). In addition, other water soluble or insoluble chemicals or drugs and/or adjuvants described above can be incorporated into the vaccine compositions of this invention. For example, muramyl dipeptides may also be used at similar ratios as described above or as desired. Other drugs which may form part of this vaccine may include any substance that when taken into the vaccinee modifies one or more of its functions, for example as recited in an official pharmacopeia, or a substance used in the treatment or prevention of an infection.

The vaccine compositions of this invention may further contain suitable carriers which are well known to those of skill in the vaccine art and can be readily selected. Exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil, squalene and water. Additionally, the carrier or diluent may

include a time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax. Optionally, suitable chemical stabilizers may be used to improve the stability of the pharmaceutical preparation. Suitable chemical stabilizers are well known to those of skill in the art and include, for example, citric acid and other agents to adjust pH, chelating or sequestering agents, and antioxidants. Alternatively, when a liposomal delivery system is part of the vaccine composition, no carriers are necessary.

Another preferred vaccine composition of this invention comprises a selected antigen, e.g., the flu D protein as described above, and 3D-MPL, which components are entrapped or intercalated in a liposome preparation. Optionally, the liposome, flu D protein, and 3D-MPL-containing vaccine composition may also contain one or more additional influenza antigens or other antigens or desirable adjuvants and agents as described above. In one preferred formulation, the vaccine contains the antigenic polypeptide Flu D protein and 3D-MPL placed into a carrier liposome. In another formulation, the vaccine contains a mono- or multi-valent influenza antigen similarly involved with a carrier liposome.

The inventors have discovered that the liposome preparations described herein are capable of functioning not only as carriers, but also as adjuvants, and are particularly advantageous because making them does not require organic solvents or high shear fields which is a significant advantage for protein drugs, and all ingredients for the liposomes are considered safe for internal administration. Because of the simplicity and flexibility of the preparative method, these liposome preparations are suitable for large scale manufacturing.

The inventors have also discovered that 3D-MPL can be readily incorporated into the liposomal structures described herein in combination with influenza antigens to obtain results superior to that found when influenza antigens are combined with known conventional adjuvants. Significantly, a vaccine composition containing D protein

in a 3D-MPL and liposome formulation has even greater potency than D protein in CFA. See, e.g., Examples 20 and 22 below.

The liposome preparations useful in the vaccine compositions and methods of this invention are described in co-pending United States Patent Application Ser. No. 07/714,984, (US 5230899) incorporated herein by reference. Generally, the word "liposome" has been proposed and accepted as the term to be used in the scientific literature to describe synthetic, oligolamellar lipid vesicles. Such vesicles are usually comprised of one or more concentric natural or synthetic lipid bilayers surrounding an internal aqueous phase.

Specifically as defined herein and according to the incorporated reference, the liposome preparations useful in the vaccine composition of the invention are prepared by dispersing in an aqueous medium in a manner adequate to form liposomes, a composition comprising a liposome-forming material containing a long chain aliphatic or aromatic-based acid or amine; a hydrating agent of charge opposite to that of the acid or amine, which agent is present in a molar ratio of between 1:20 and 1:0.05 relative to the acid or amine; and water in an amount up to 300 moles relative to the solids.

The preparation of the liposomal adjuvant carriers useful in the invention follows. Examples of liposome-forming materials include saponifiable and non-saponifiable lipids, e.g., the acyl glycerols, the phosphoglycerides, the sphingolipids, the glycolipids, etc. The fatty acids include saturated or unsaturated alkyl (C_8~C_{24}) carboxylic acids, mono-alkyl (C_8~C_{27}) esters of C_4~C_{10} dicarboxylic acids (e.g., cholesterol hemi-succinic acid and fatty acid derivatives of amino acids in which any N-acyl carboxylic acids also are included (e.g., N-oleoyl threonine, N-linoleoyl serine, etc.). Mono- or di-alkyl (C_8~C_{24}) sulfonate esters and mono- or di-alkyl (C_8~C_{24}) phosphate esters can be substituted for the fatty acids. Furthermore, mono- or di-acyl (C_8~C_{24}) glycerol derivatives of phosphoric acids and mono- or di-

acyl (C_8~C_{24}) glycerol derivatives of sulfuric acids can be used in place of the fatty acids.

Additionally, the fatty acids also can be replaced by amines (e.g., C_8~C_{24} NH_2), C_8~C_{24} fatty acid derivatives of amines (e.g., C_8~C_{24} CONH~NH_2), C_8~C_{24} fatty alcohol derivatives of amino acids (e.g., C_8~C_{24} OOC~NH_2), and C_8~C_{24} fatty acid esters of amines (e.g., C_8~C_{24} COO~NH_2).

Photopolymerizable lipids and/or fatty acids (or amines) (e.g., diacetylenic fatty acids) also can be included, which can provide a sealed liposome with cross-linked membrane bilayers upon photo-initiation of polymerization.

A long chain aliphatic and/or aromatic-based acid or amine includes an acid or amine having an open chain structure and consisting of paraffin, olefin and acetylene hydrocarbons and their derivatives, i.e., saturated and unsaturated hydrocarbons or the backbone of such chain contains an where aromatic substituent. Such acids and amines may have more than one such function. The term "long chain" means that the backbone of the aliphatic chain of the acid or amine has ten or more carbon atoms. If the chain contains an aromatic group, such as phenyl, the chain will comprise at least a five carbon backbone in conjunction with that aromatic group. The chain of carbon atoms comprising the backbone may be variously substituted with saturated or unsaturated aliphatic or aromatic functions.

The terms "acid" or "amine" are conventionally defined chemical functionalities. For example, an acid function may be a carboxylate acid, or a phosphorous or sulfur derived acid function such as phosphate, phosphite or pyrophosphate or sulfate, sulfite, thiosulfate, or similarly constituted phosphorous or sulfur-based acid. Amines must be sufficiently basic so as to have an ionizable hydrogen or be capable of forming quaternary salts which have an ionization constant such that they are capable of forming the requisite hydrate complex.

As used in the vaccine composition and relative to the amount of water employed in the liposome composition,

the term "solids" refers to the liposome-forming materials and the acid or amine component, hydrating agents, the 3D-MPL, alum, and the selected antigenic material to be encapsulated.

For the purpose of this invention, a hydrating agent means a compound having at least two ionizable groups, preferably of opposite charge, one of which is capable of forming an easily dissociative ionic salt, which salt can complex with the ionic functionality of the acid or amine in the liposome-forming material. The hydrating agent inherently does not form liposomes in and of itself. Such an agent will also be physiologically acceptable, i.e., it will not have any untoward or deleterious physiological effect on the host to which it is administered in the context of its use in this invention.

The preferred hydrating agents are alpha amino acids having an ionizable omega substitution such as a carboxylate, amino, and guanidino function and those compounds represented by the formula:

$X-(CH_2)n-Y$ I

wherein

X is $H_2N-C(NH)-NH-$, H_2N-, ZO_3S-, Z_2O_3P-, or ZO_2C- wherein Z is H or an inorganic or organic cation;

Y is $-CH(NH_2)-CO_2H$, $-NH_2$, $-NH-C(NH)-NH_2-COOH$, $CH(NH_2)SO_3Z$ or $ZH(NH_2)PO_3Z_2$ wherein Z is defined above; and

n is the integer 1-10; or

a pharmaceutically acceptable salt thereof.

Also included in the list of preferred compounds are the N,N'-dialkyl substituted arginine compounds and similar compounds where the alkyl chain length is varied.

More preferred hydrating agents are the omega-substituted, alpha amino acids such as arginine, its N-acyl derivatives, homoarginine, gamma-aminobutyric acid, asparagine, lysine, ornithine, glutamic acid, aspartic acid or a compound represented by the following formulas:

$H_2NC(NH)-NH-(CH_2)_n-CH(NH_2)COOH$ II

$H_2N-(CH_2)_n-CH(NH_2)COOH$ III

$H_2N-(CH_2)_n-NH_2$ IV

$H_2NC(NH)-NH-(CH_2)_n-NH-CH(NH)-NH_2$ V

$HOOC-(CH_2)_n-CH(NH_2)COOH$ VI

$HOOC-(CH_2)_n-COOH$ VII

$HO_3S-(CH_2)_n-CH(NH_2)COOH$ VIII

$H_2O_3S-(CH_2)_n-CH(NH_2)COOH$ IX

$HO_3S-(CH_2)_n-CH(NH_2)SO_3H$ X

$H_2O_3S-(CH_2)_n-CH(NH_2)PO_3H_2$ XI

wherein n is 2-4.

The most preferred compounds are arginine, homoarginine, gamma-aminobutyric acid, lysine, ornithine, glutamic acid or aspartic acid. The hydrating agents of this invention may be used alone or as a mixture. No limitation is implied or intended in the use of mixtures of these hydrating materials.

The hydrating agents of this invention are listed in the catalogue of many chemical producers, can be custom manufactured by such producers, or can be made by means known in the art. Arginine, homoarginine, lysine, glutamic acid, aspartic acid, and other naturally occurring amino acids may be obtained by the hydrolysis of protein and separation of the individual amino acids or from bacterial sources.

The compounds of formula II can be made by the method of Eisele, K. et al, Justusliebigs. Ann. Chem., p. 2033 (1975). Further information on several representative examples of these compounds is available through their respective Chemical Abstracts Service numbers as follows: nonarginine, CAS #14191-90-3; arginine, CAS #74-79-3; and homoarginine, CAS #151-86-5. For representative examples of formula III, see for 2,4-diaminobutonic acid CAS #305-62-4 and for lysine CAS #56-87-1. Methods for making representative compounds of formula IV are available from Chemical Abstracts as follows: ethane diamine, CAS #305-62-4; propane diamine - 54618-94-9; and 1,4-diaminobutane, CAS #52165-57-8. See specifically Johnson, T.B., J. Am. Chem. Soc., 38:1854 (1916).

Of the compounds of formula VI, glutamic acid is well known in the art and is available from many commercial sources. Descriptions of how to make other

representative compounds is contained in the literature, for example: 2-aminohexandioc acid - CAS # 62787-49-9 and 2-aminoheptandioc acid - CAS # 32224-51-0. Glutamic acid, the compound of formula VII where n is 2 is well known in the art and can be made by the method of Maryel and Tuley, Org. Syn., 5:69 (1925). Other representative compounds in this group can be made according to the art as referenced by the following CAS numbers: hexadioic acid, CAS # 123-04-9 and heptadioic acid, CAS # 111-16-0. Homocysteic acid is known in the art referenced by CAS # 56892-03-6. The compound 3-sulfovaline is described in the literature referenced by CAS #23405-34-2.

Mixtures of the hydrating agent, liposome-forming materials and discrete amounts of water form a gel-like mass. When in this gel form, the hydrating agent and acid or amine, in conjunction with all liposome forming materials, arrange into a "hydrate complex" which is a highly ordered liquid crystal. Hydrate complex means the complex formed between the hydrating agent and the acid or amine in the liposome-forming material. Complexing in this context denotes the formation of dissociative ionic salts where one functionality associates with the ionic functionality of the liposome-forming material and the other functionality has hydrophilic properties which impart water-solubility to the resulting complex. While the liquid crystal structure of the hydrate complex varies with pH and amount of hydrating agent, the liquid crystal structure remains. NMR spectroscopy confirms that this crystal structure consists of multilamellar lipid bilayers and hydrophilic layers stacked together in alternating fashion. The ^{31}P-NMR spectrum exhibits an anisotropic peak, confirming the existence of multilamellar bilayers.

Although the primary components of these liposomes will be lipids, phospholipids, other fatty acids, there may also be added various other components to modify the liposomes' permeability. There may be added, for example, non-ionic lipid components such as polyoxy alcohol compounds, polyglyceral compounds or esters of

polyoles, polyoxyalcolinolated alcohols; the esters of polyoles and synthetic lipolipids, such as cerebrosides. Other materials, such as long chain alcohols and diols, sterols, long chain amines and their quaternary ammonium derivatives; polyoxyethylenated fatty amines, esters of long chain amino alcohols and their salts and quaternary ammonium derivatives; phosphoric esters of fatty alcohols, polypeptides and proteins.

The composition of the liposome can be made of more than one component of the various kinds of lipids, the fatty acids, alkyl amines, or the like, and the hydrating agents.

If the lipid component itself or antigenic material and any other materials to be added to the vaccine composition to be encapsulated possess the aforementioned properties, the lipid composition may not require the inclusion of the fatty acids (or the amines) or the hydrating agents to form the "pre-liposome gel" or liposomes. For example, the mixture of dipalmitoylphosphatidylcholine (DPPC) and distearoyl phosphatidylethanolamine forms the "pre-liposome gel" or liposomes with aqueous glutamic acid solution and the mixture of DPPC and oleic acid with aqueous epinephrine solution forms the "pre-liposome gel" and liposomes.

For use in the vaccine composition as an adjuvanting material, the liposome preparation preferably includes phospholipids, oleic acid (or phosphatidyl-ethanolamine) and arginine or lysine (or glutamic acid and/or aspartic acid).

About 1:20 molar ratio of hydrating agent relative to the liposome-forming material will provide the salutory effects of this invention with an upper limit of about 1:0.05. The preferred concentration range for the hydrating agent is between a 1:2 to 1:0.5 molar ratio of the hydrating relative to the liposome-forming material.

As a practical matter, thus a matter of preference, if liposomes are prepared with a long chain aliphatic or aromatic-based acid, it is preferred to use hydrating agents which contain at least one ionizable nitrogen,

such as arginine, homoarginine, lysine, ornithine, and the like. Conversely, if the amphipatic materials used to form the liposomes contain a long chain aliphatic or aromatic-based amine, it is preferred to use a di-acid such as glutamic acid, aspartic acid; any of the alkyl di-acids such as the simple di-acids such as valeric acid, caprylic, caproic, capric or the like; or those di-acids having two phosphate, or sulfate functionalities; or those di-acids having mixed -COOH/-SO_3H or -COOH/-PO_3H_2 functions.

Certain aliphatic and aromatic-based acids and amines are preferred in the practice of this invention. Such compounds can have multiple functions such as having two or more acid or amine groups or combinations thereof. For example, one could use a di-acid, a di-amine or a compound having an acid and an amine function. The preferred compounds are those with one or two acid or amine functions. More preferred are the fatty mono-acids of 10-20 carbons, saturated and unsaturated. Most preferred are the alkyl and alkenyl acids of 10 to 20 carbon atoms, particularly oleic acid.

Mixtures of liposome-forming materials, a long chain aliphatic or aromatic-based acid or amine, one or more hydrating agents with up to 300 moles of water relative to the total solids, with or without a selected amount of antigenic material, produces a gel which forms liposomes directly therefrom upon addition of an aqueous solution. This gel can be labelled a pre-liposome gel because its structural characteristics are essentially those of liposomes and, the gel has the facility for being converted into liposomes upon dilution with an aqueous solution. Aqueous solution in excess of about 300 molar equivalents cause the beginning of liposome formation.

The structure of this gel is a highly ordered liquid crystal which forms an optically clear solution. The X, Y, and Z dimensions of the liquid crystal vary with the concentrations of hydrating agent at constant pH as well as with the pH of the solution. By varying the hydrating agent concentration at constant pH or changing the pH

while maintaining the percentage of hydrating agent, the size and number of lamellae structures of the lipid bilayers of the subsequent liposome vesicles can be controlled.

The gel structure itself can accommodate up to approximately 300 moles of water per mole of solid without disturbing the stability of the gel structure. The structure of the gel as determined by proton nuclear magnetic resonance (NMR) spectroscopy is comprised of multilamellae lipid bilayers and hydrophilic layers stacked together in an alternating fashion. The ^{31}P-NMR spectrum of the same gel exhibits an anisotropic peak further confirming that the gel consists of a multilamellar bilayer. This gel can be autoclaved, a convenient means of sterilization. Furthermore, the gel shows no discoloration and remains clear at room temperature for at least one year after being autoclaved. If desirable and feasible, the gel can optionally further be sterilized by filtration through an appropriate sterilization filter. Upon dispersion of the gel into an aqueous solution, liposomes are efficiently and spontaneously produced.

The pre-liposome gel, with or without the 3D-MPL and antigenic material to be encapsulated, also can be dehydrated (e.g. lyophilized) and the powder rehydrated to form liposomes spontaneously, even after a long period of storage. This capability provides the vaccine compositions particularly useful for administering water-sensitive antigenic materials where long term pre-use storage is needed.

The pre-liposomal gel is prepared as follows. A semisolid liquid crystalline gel referred to as the pre-liposomal gel is prepared by combining three basic ingredients: a phospholipid, a fatty acid, and a hydrating agent in water. Depending upon the lipid composition desired, a variety of phospholipids or mixtures thereof ranging in gel-liquid transition temperatures (Tm) may be employed. The fatty acid can be chosen based on degree of saturation or chain length and

is usually mixed with the phospholipid into a thick paste. Cholesterol may be added to the lipid mix to control the bilayer character. (Cholesterol in some instances increases the Tm of the membrane thereby influencing its permeability to entrapped agents).

The hydrating agent, preferably an alpha amino acid, such as arginine, is added to the lipid mix as a solution in water at a slow rate until a homogeneous paste is achieved. One desirable formulation employs egg phosphatidyl choline:oleic acid at a 1:1.2 molar ratio. The arginine is added in water at approximately 1:1.2 phospholipid to arginine molar ratio.

The concentration of L-arginine in the aqueous phase component of the gel governs the diameter of the stable liposome particle which eventually forms. The size of the particle depends on the route of administration and whether or not it is desired to target macrophage cells. For example, for oral administration, a liposome particle size of between about 1 to about 5 micrometers is desired. To target to lymphocytes, a particle size of between about 200 to 500 nm is preferred. For a depot effect, a liposome particle size between about 5 to about 10 micrometers is desired. Various size particles may be easily tested and selected by one of skill in the art.

The final pre-liposomal gel can contain up to about 65 to about 70% water by weight, has the consistency of an ointment, and has the appearance of a typical liquid crystal when observed under a polarizing light microscope. The pre-liposomal gel can be stored under an inert atmosphere, or lyophilized to a dry powder for long term storage.

In the formation of the vaccine compositions of this invention, the same steps of liposome preparation are followed with the addition of an immunologically effective amount of the selected antigen, e.g., an antigenic polypeptide, particularly the flu D protein, and 3D-MPL, and optionally, one or more additional immunogenic proteins, peptides or fragments from a selected pathogen mixed with the liposome preparation.

To prepare such vaccine compositions, the selected antigen is encapsulated or intercalated within the liposome preparation by mixing therewith.

There are two methods by which the 3D-MPL and the selected antigen can be incorporated into the liposome composition to prepare a vaccine composition according to this invention. One method involves the incorporation of the antigen with liposomes by hydration of the pre-liposomal gel, or the hydrated lyophilized powder, with a solution of the antigen in an appropriate buffer. The other method involves the physical mixing of the pre-liposomal gel with a lyophilized preparation of the antigen. This mixture is subsequently hydrated which causes the spontaneous formation of liposomes.

The method selected is the one which results in the largest level of antigen-liposome association with the smallest loss of antigen as unassociated fraction and is dependent upon the physico-chemical properties of the antigen in the presence of the lipid. Generally, however, the ratios of the components in such a vaccine mixture are 20 mg antigenic protein: 2 mg 3D-MPL: 300 mg liposomal gel. For instance, when the antigen is flu D, the second method is employed because the antigen has significant hydrophobic character and a low aqueous solubility and, as below described, to form a flu-D composition according to this invention, 300 mg of pre-liposomal gel was mixed with 20 to 30 mg flu-D protein and 0.5 to 2 mg 3D-MPL.

It is considered that one of skill in the art given the teachings herein can readily determine the selected amount of the antigenic material to use in the practice of this invention.

Optionally, other agents may be added co-entrapped with the selected antigen, e.g., flu D protein, and 3D-MPL in a liposome composition. Suitable other agents are described above.

Once the liposomes are formed in the presence of the 3D-MPL, selected antigen, and any optionally additional components, any unassociated antigen may be removed by

various means. Typically, the liposomes are centrifuged serially at approximately 100,000 x g and the supernatants are discarded. The final liposome pellet is reconstituted in an acceptable liquid, for example, 5% dextrose, normal saline, or buffered solution, for injection at a desired lipid or antigen concentration. Other methods known to those of skill in the art, such as gel filtration or dialysis, may be employed for removal of unassociated antigen.

The description of the selected antigen, 3D-MPL and liposome formulation also encompasses the use of the split viruses described herein in place of, or in addition to, Flu D.

The present invention also provides a method of enhancing an immune response, particularly a T-cell mediated response, in a human or other mammal, to the selected antigen in the vaccine composition. This method involves administering to the human or other mammal a vaccine composition of the invention containing 3D-MPL and the selected antigen. Optionally a liposome preparation may be part of the composition as described above. This method is not limited to any particular antigen exemplified herein. In a preferred embodiment, the method is useful in eliciting an enhanced protection effective against influenza infection. In such an embodiment, the vaccine composition comprises an effective amount of flu D and 3D-MPL, as described above. In another embodiment the vaccine composition comprises an effective amount of one or more split influenza viruses and 3D-MPL, as described above. Other vaccines may be prepared and administered in effective amounts in a similar manner.

As indicated by the examples, particularly Examples 25-30, the vaccine composition employing split viruses adjuvanted with 3D-MPL results in superior virus clearance particularly in the lung, the stimulation of higher neutralizing antibody titers; the occurrence of heterosubtypic (H_1N_1) cross-reactivity in the absence of neutralizing activity; and altered progression of disease

from upper to lower respiratory track (via trachea).

The dosage and administration protocols can be optimized in accordance with standard vaccination practices for these vaccine compositions. Typically, the vaccines will be administered intramuscularly, although other routes of administration may be used, such as the subcutaneous, colonic, oral, pulmonary, intradermal, intraperitoneal, or intravenous routes. The route chosen may be dictated by the type of immune response desired. For example, a subcutaneous route may provide the classical "depot effect" or more prolonged stimulation than others. It is also possible that a given route of administration may generate a stronger antibody response than a cellular response, or vice versa. For example, the oral route of administration may permit generation of an enhanced IgA response that is useful for local immunity.

Based on what is known about other polypeptide vaccines, it is expected that a useful single dosage for average adult humans of an influenza antigenic polypeptide vaccine, such as the flu D protein containing vaccine of this invention is in the range of between about 1 to about 1000 micrograms of D protein, preferably about 50 to about 500 micrograms protein, and most preferably about 100 µg, in admixture with suitable amounts of 3D-MPL adjuvant. When these doses of flu D protein are used, the amounts of 3D-MPL in the vaccine composition are between about 1 to about 500 micrograms 3D-MPL/µg viral protein, more preferably about 10 to about 50 µg 3D-MPL/µg viral protein, and most preferably about 50 µg 3D-MPL.

When flu D and 3D-MPL are administered via a liposome carrier as described herein, the preferred amount of D protein in the vaccine composition is between about 50 to about 500 µg and the amount of 3D-MPL is between about 10 to about 50 µg. Such a 3D-MPL adjuvanted flu D vaccine composition will contain about 1 to about 10 mg, and preferably about 3 mg, liposome material per about 0.2 mg antigenic protein.

When the flu D and 3D-MPL vaccine formulation optionally contains another adjuvant, such as aluminum or aluminum hydroxide, the preferred dosage of D protein is between about 10 to about 500 µg protein; the preferred amount of aluminum or aluminum hydroxide is between about 10 to about 500 µg.

Similarly, based on what is known about other split virus vaccines, it is expected that a useful single dosage for average adult humans of an influenza monovalent or multivalent split virus vaccine, such as those described herein, is about 15 micrograms of hemagglutinin (HA) per strain, with total protein ranging from about 80-300 µg/ml, in admixture with suitable amounts of 3D-MPL adjuvant. When these doses of split virus are used, the amount of 3D-MPL in the vaccine composition is preferably about 50 micrograms 3D-MPL per dose. Of course, these amounts of virus protein and 3D-MPL may be altered by one of skill in the art.

When one or more split virus and 3D-MPL are administered via a liposome carrier as described herein, the preferred amount of each split virus in the vaccine composition is likely to be less than about 15 µg HA/strain and the amount of 3D-MPL is between about 10 to about 50 µg. Such a 3D-MPL adjuvanted influenza split virus vaccine composition will contain about 1 to about 10 mg, and preferably about 3 mg, liposome material per about 0.2 mg viral protein.

When the split virus and 3D-MPL vaccine formulation optionally contains another adjuvant, such as aluminum or aluminum hydroxide, the preferred dosage of each split virus in the vaccine composition may be adjusted downward. A preferred amount of aluminum or aluminum hydroxide is similar to that described above for the antigenic polypeptide.

Any of the vaccines described by this invention can be administered (preferably in 0.5 mls dosage units) initially in late summer or early fall and can be readministered two to six weeks later, if desirable, or periodically as immunity wanes, for example, every two to

five years.

The following examples illustrate the preferred methods for preparing vaccine compositions of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Gel Preparation

Dipalmitoylphosphatidylcholine, 3.0 grams, was weighed into a 50 ml beaker. Oleic acid 1.2 grams was added and mixed together to form a uniform paste.

Arginine 0.72 grams in 30 ml of distilled deionized water was added to the dipalmitoylphosphatidyl-cholineoleic acid paste and heated to 45°C. With mixing by hand, the mixture formed a clear stable gel. The gel was stored and liposomes later formed by diluting the get with phosphate buffered saline.

Example 2 - Preparation of Liposomes

Dipalmitoylphosphatidylcholine, 120 mg, and 24 mg of oleic acid were added together and mixed thoroughly until a white homogeneous paste was observed.

Then 20 mg of arginine was dissolved into 60 ml of phosphate buffered saline (ionic strength = 0.15, pH = 7.4). The arginine-saline solution was added to the paste and heated to 40°C for ½ hour, or until a slightly turbid solution was observed.

Example 3 - Large Scale Gel and Liposome Preparation

i) Gel Manufacture: To 50 grams of egg phosphatide powder type 20 (Asahi Chemicals) was added 20 grams of oleic acid N.F. Mixing gave a white paste which was cooled to 4°C and ground into a fine powder. This powder was added to an aqueous solution containing 20 grams of arginine and 500 grams of distilled deionized water. The mixture was mixed with a spatula as the solution was heated to about 35°C to help hydrate phospholipids. A homogeneous, slightly yellow get was formed. This gel can be autoclaved and stored at 4°C or can be frozen and later reconstituted.

ii) Manufacture of Liposomes: The gel prepared in the preceding paragraph was taken from cold storage and returned to room temperature. It was then mixed with 2 liters of phosphate buffered saline, pH 7.4. A white opaque liposome solution was formed.

Example 4 - Liposome Formation from the Gel

A homogenous paste of 1.0 gram of dipalmitoylphosphatidycholine (DPPC) and 400 mg of oleic acid was formed. Then 300 mg arginine was mixed in 10 mL of phosphate buffered saline, heated to 45°C and added to the DPPC/oleic acid paste to form liposomes.

Example 5 - Pre-Liposome Gel

One gram of dipalmitoylphosphatidylcholine (DPPL) was mixed with 400 mg of oleic acid to form a homogeneous paste. 300 mg of arginine was mixed with 2 ml of water at 45°C until dissolved. The arginine solution was mixed with the DPPC/oleic acid paste at about 45°C to give a thick gel. Liposomes formed when this gel was diluted with phosphate buffered saline.

Example 6 - Various Liposome Formulations

A. Cholesterol Containing Liposomes

Cholesterol, 15 mg, was mixed with 100 mg dipalmitoylphosphatidylcholine (DPPC) to form a homogeneous powder. Then 23 mg of oleic acid was added to the powder and thoroughly mixed to form a homogeneous paste. To make liposomes, 30 mg of arginine was added to 10 ml of phosphate buffered saline, heated to 40°C and added to the DPPC/cholesterol/oleic acid paste. The combination was mixed at 40°C to obtain liposomes.

B. Palmitic Acid-Containing Liposomes

Dipalmitoylphosphatidylcholine (DPPC) 250 mg was mixed with 25 mg of palmitic acid to form a uniform powder. Then 80 mg of oleic acid was mixed with this powder and heated to 45°C with constant stirring until a uniform paste was formed. Arginine 100 mg was dissolved in 25 ml of distilled deionized water and heated to 45°C.

This arginine solution was added to the paste at 45°C and mixed until a uniform homogeneous gel was formed. The gel was diluted ten fold with phosphate buffered saline to form liposomes.

C. Isostearic Acid-Containing Liposomes

Dipalmitoylphosphatidylcholine 100 mg was mixed with 50 mg of isostearic acid to form a uniform homogeneous paste. An arginine solution of 50 mg of arginine in 2.0 ml of distilled deionized water was made and added to the isostearic acid paste and heated to 45°C. This mixture was mixed until a clear gel was formed. Liposomes are formed upon dilution with phosphate-buffered saline.

D. Oleoyl Threonine Containing Liposomes

Dipalmitoylphosphatidylcholine 125 mg and 75 mg of oleoyl threonine were added together and heated to 40°C to form a paste. Then 2 ml of distilled deionized water was added with constant mixing at 40°C.
A clear gel was formed which can be diluted with phosphate buffer saline at pH 5 to form liposomes.

E. Myristyl Amine containing Liposomes

Dipalmitoylphosphatidylcholine 192 mg was added to 72 mg of myristyl amine and heated with constant mixing until a uniform paste was formed. Glutamic acid 65 mg in 5 ml of distilled deionized water was added to the paste and heated until a gel was formed. Phosphate buffered saline was added to the get to form liposomes.

F. DLPC Containing Liposomes

Dilaurylphosphatidylcholine (DLPC) 50 mg was mixed with 20 mg oleic acid to form a homogeneous paste. Arginine 20 mg was added to 10 ml of phosphate buffered saline, added to the paste and hand mixed until a turbid liposome solution formed.

G. Phosphatidylethanolamine-Glutamic Acid Liposomes

L-glutamic acid 32 mg was dissolved in 2.0 ml of distilled deionized water and the pH adjusted to 5.2 with 1.0 N sodium hydroxide. This solution was heated to 60°C, and 100 mg of phosphatidylethanolamine added. The solution was kept to 60°C with constant mixing until a uniform viscous gel was observed.

The phosphatidylethanolamine-glutamic acid gel was diluted 1/10 by phosphate buffered saline. Vesicular-like structures are observed under phase contrast light microscopy.

Example 7 - Effect of Arginine Concentration on Liposome Size

To 502 mg of dipalmitoylphosphatidylcholine (DPPC) was added 10 microliters of (2-palmitoyl-1-C_{14}) (0.1 mCi/ml) dipalmitoylphosphatidylcholine. Chloroform was added to effect complete mixing of the radioactivity and then evaporated. Oleic acid (OA), 195 mg, was then mixed into the lipid to form a paste. Five ml of distilled water containing 119 mg of arginine was added and mixed at 45°C to form a clear gel.

One gram of the gel was weighed into four different vials and arginine was added as indicated in the following Table 1.

Table 1

Sample ID	Sample Composition DPPC:OA:Arg
Vial 1 + 1 ml water	(1:1:1)
Vial 2 + 1 ml of 50 mg/ml Arg in H_2O	(1:1:3)
Vial 3 + 1 ml of 84 mg/ml Arg in H_2O	(1:1:5)
Vial 4 + 1 ml of 192 mg/ml Arg in H_2O	(1:1:10)

One-half gram of each solution was diluted in 50 ml of phosphate buffered saline of pH 7.8.

The estimated average diameter was obtained from a Sephracyl S-1000 column chromatographic analysis employing $_{14}C$-isotope labelled DPPC (i.e. diameters were determined based on intensity of scattering using photon correlation spectroscopy (PCS)). The effects are given

in the following Table 2.

Table 2

Effects of Arginine Concentration on Vesicle Size

System	Ratio	pH	Estimated Diameter (nm)
DPPC:OA:Arg	1:1:1	7.8	~220
DPPC:OA:Arg	1:1:3	7.8	~140
DPPC:OA:Arg	1:1:5	7.8	~90
DPPC:OA:Arg	1:1:10	7.8	~20

Example 8 - pH Effect on Vesicle Size

Additionally, the vesicle size can be varied by varying the pH of the aqueous buffer solution.

To 100 mg of dipalmitoylphosphatidylcholine (DPPC) was added 25 microliters of (2-palmitoyl-1-C_{14}) (0.1 mCi/ml) dipalmitoylphosphatidylcholine. Chloroform was added to effect complete mixing of the radioactivity and then evaporated. Oleic acid (OA), 40.1 mg, was then mixed into the lipid to form a paste. One ml of a solution containing 24 mg/ml arginine in water was added to the lipid mixture and mixed at 45°C to form a clear gel.

Two 100 mg aliquots of this gel were diluted in 10 ml of phosphate buffer at pH 9.0 and 7.4 respectively.

Again, the estimated average diameter was obtained from the Sephracyl S-1000 column chromatographic analysis employing ^{14}C-isotope labelled dipalmitoyl-phosphatidylcholine. Results are given in the following Table 3.

Table 3

pH Effects on Vesicle Size

System	Ratio	pH	Estimated Diameter (nm)
DPPC:OA:Arg	1:1:1	7.4	~<300
DPPC:OA:Arg	1:1:1	7.8	~220
DPPC:OA:Arg	1:1:1	9.0	~25.4

Thus, a desired size of the liposomal vesicles can be prepared by varying the arginine concentration or the pH of the aqueous buffer solution.

Example 9 - Liposome Stability

Sterile liposomes may be prepared from the heat sterilized pre-liposome gel. Alternatively, the liposome gel or the liposomes may be sterile filtered through an appropriate sterilizing filter.

Liposomes prepared from DPPC:OA:Arg (1:1:2) at pH 8.0 were heat sterilized and stored at room temperature for approximately one year without adding antimicrobial agents and anti-oxidants. No bacterial growth, discoloration and precipitation were observed. Negative stain electron microscopic examination of the one year old liposomes revealed that the liposomal vesicles are stable.

Example 10 - Sucrose Latency

Encapsulated sucrose latency was measured using C_{14}-sucrose encapsulated with the DPPC:OA:Arg (1:1:1) liposome system in aqueous phosphate buffer solution at pH 7.8. The result was presented in the following Table 4.

Table 4

Days	% Sucrose Latency
0	100
1	97.4
3	93.4
7	91.4

Thus, the present liposome system has an excellent latency for drug delivery.

Example 11 - Lyophilized Liposomes

Oleic acid, 30.0 gm, and 7.5 gm of cholesterol U.S.P. were confected. Then 75.0 gm of phosphatide type 20 powder (Asahi Chemical Co.) was mixed with the oleic acid/cholesterol mixture until an homogeneous paste was formed.

Then 15.0 gm of arginine (free base) was dissolved in 183 gm of distilled, deionized water. This arginine solution was mixed slowly with the lipid paste to form a homogeneous gel. The gel pH was adjusted to 7.4 using 5.0 N HCl.

A 10.0 gm, aliquot of this pre-liposome gel was transferred to a 10 ml vial and lyophilized. The resulting powder formed liposomes when diluted with 5 ml of phosphate buffered saline.

Example 12 - Construction of Flu D Expression Plasmids

Plasmid pAPR701 is a pBR322-derived cloning vector which carries coding regions for the M1 and M2 influenza virus proteins (A/PR/8/34). It is described by Young et al, in The Origin of Pandemic Influenza Viruses, 1983, edited by W. G. Laver, Elsevier Science Publishing Co.

Plasmid pAPR801 is a pBR322-derived cloning vector which carries the NS1 coding region (A/PR/8/34). It is described by Young et al, cited above.

Plasmid pAS1 is a pBR322-derived expression vector

which contains the P_L promoter, an N utilization site (to relieve transcriptional polarity effects in the presence of N protein) and the cII ribosome binding site including the cII translation initiation codon followed immediately by a BamHI site. It is described by Rosenberg et al, Methods Enzymol., 101:123-138 (1983).

Plasmid pAS1deltaEH was prepared by deleting a non-essential EcoRI-HindIII region of pBR322 origin from pAS1. A 1236 base pair BamHI fragment of pAPR801, containing the NS1 coding region in 861 base pairs of viral origin and 375 base pairs of pBR322 origin, was inserted into the BamHI site of pAS1deltaEH. The resulting plasmid, pAS1deltaEH/801 expresses authentic NS1 (230 amino acids). This plasmid has an NcoI site between the codons for amino acids 81 and 82 and an NruI site 3' to the NS sequences. The BamHI site between amino acids 1 and 2 is retained.

Plasmid pAS1ΔEH/801 was cut with BglII, end-filled with DNA polymerase I (DNApolI; Klenow) and ligated closed, thus eliminating the BglII site. The resulting plasmid pBgl$^-$ was digested with NcoI, end-filled with DNApolI (Klenow) and ligated to a BglII linker. The resulting plasmid, pB4, contains a BglII site within the NS1 coding region. Plasmid pB4 was digested with BglII and ligated to a synthetic DNA linker as described in Example 4 of EP 0366238.

The resulting plasmid, pB4+, permits insertion of DNA fragments within the linker following the coding region for the first 81 amino acids of NS1 followed by termination codons in all three reading frames. Plasmid pB4+ was digested with XmaI (cuts within linker), end-filled (Klenow) and ligated to a 520 base pair PvuII/HindIII, end-filled fragment derived from the HA2 coding region. The resulting plasmid, pD, codes for a protein comprised of the first 81 amino acids of NS1, three amino acids derived from the synthetic DNA linker (Gln-Ile-Pro), followed by amino acids 65-222 of the HA2, as shown in Fig. 2 of published European Patent Application No. 366,238.

To facilitate plasmid selection in production fermentations, a BamHI fragment derived from the pD expression plasmid encoding the recombinant flu D protein, was ligated into the BamHI site of a pAS1 plasmid derivative containing a kanamycin resistance gene from Tn903 for selection [Berg et al, Microbiology, ed. D. Schlessinger, pp. 13-15, American Society for Microbiology (Washington, DC 1978); Nomura et al, The Single-Stranded DNA Phages, ed. D. Denhardt et al, pp.467-472, Cold Spring Harbor Laboratory (New York 1978); Castellazzi et al, Molecul. Gen. Genet., 117:211-218 (1982)]. This results in the vector, $pC_{13}(H_{65-222})Kn$.

As described in Shatzman and Rosenberg, Meth. Enzymol., 152:661-673 (1987), pOTS207 is a pAS derived cloning vector which carries the kanamycin resistance gene from Tn903 [Berg, cited above; Nomura, cited above; Castellazzi, cited above]. It was constructed by digesting plasmid pUC8 [Yanisch-Perron et al, Gene, 33:103-119 (1985)], with BamHI and ligated to a BclI fragment containing the kanamycin gene from Tn903. The resulting plasmid, pUC8-Kan, was digested with EcoRI and PstI, and the fragment containing the kanamycin gene was inserted between the EcoRI and PstI sites of pOTSV [Shatzman and Rosenberg, cited above]. The resulting plasmid is pOTS207.

A 520 bp fragment encoding the HA2 coding sequence was isolated from pJZ102 [a pBR322-derived cloning vector which carries a coding region for the entire HA protein (A/PR/8/34) (described by Young et al, The Origin of Pandemic Influenza Viruses, ed. W.G. Laver, Elsevier Science Publishing Co. (1983)] and inserted into pB4+ which had been cut with XmaI and end filled. The resulting plasmid, $pC_{13}H_{65-222}$ was digested with BamH1 and the fragment encoding the flu D protein isolated from this fragment was then ligated into the BamH1 site of pOTS207 to produce the plasmid $pC_{13}(H_{65-222})Kn$ [SmithKline Beecham].

The sequence of the kanamycin resistant C13(H65-222)Kn plasmid was derived as follows: nucleotide

positions 1-31, 3136-3964, 4021-4343, 4533-7166 were derived from pBR322 [Young et al, cited above]; nucleotide positions 32-1879, 4344-4532 were derived from λ phage, nucleotide positions 1880-2122, 2682-3135 were derived from the NS1 gene, nucleotide positions 2123-2132, 2660-2681 were derived from a synthetic linker, nucleotide positions 2133-2659 were derived from the HA2 gene, nucleotide positions 3965-4020 were derived from the pCV_1 polylinker, and nucleotide positions 7167-8601 were derived from the pUCKan12 ($Tn903:Kn^r$). The DNA sequence of the coding region for the flu D protein derivative was confirmed by the dideoxy chain termination method of sequencing DNA [Sanger et al, Proc. Natl. Acad. Sci. USA, 74:5463-5467 (1977)].

This $C_{13}(H_{65-222})Kn$ plasmid is essentially the same as the plasmids which are described in co-pending U. S. Patent Application Serial No. 07/387,200, its corresponding published EPA No. 366,238, and co-pending U. S. Patent Application Serial No. 07/387,558, its corresponding published EPA No. 366,239, with the exception that the β-lactam marker has been removed and replaced with a Kanamycin marker. The above applications are incorporated for reference for their description of other appropriate vectors.

The $pC_{13}(H_{65-222})Kn$ plasmid was transformed into *E. coli* expression strain AR58 [SmithKline Beecham]; and production of the flu D protein was confirmed by immunoblot analysis [Towbin et al, Proc. Natl. Acad. Sci. USA, 76:4350 (1979)] which revealed a major immunoreactive species at the predicted molecular weight of 27.7 kD.

Example 13 - Purification of D Protein

The $NS1_{1-81}$-$HA2_{65-222}$ or D protein (MW 27.7 kD), was purified as described in detail in published European Patent Application N0. 366,239, corresponding to U.S. Patent Application Ser. No. 07/387,558.

Following the induction of synthesis of D protein by any *E. coli* host strain AR58 [SmithKline Beecham] using

the pC13(H_{65-222})Kn system described in Example 12, the bacterial cells were collected by centrifugation and frozen at -70°C until used. This pellet was thawed and resuspended in 50 mM Tris, 2 mM EDTA, 0.1 mM dithiothreitol (DTT), 5% glycerol, at pH 8. The resulting suspension was treated with lysozyme (final concentration of at least about 0.2 mg/ml) for about 1 hour under ambient conditions.

This suspension was then lysed on a Manton Gaulin homogenizer [APV Gaulin, Inc., Everett, Massachusetts] at 8,000 psi in two passes. The resulting suspension was treated with Triton X-100 (1% final concentration) and deoxycholate (DOC; 0.1% final concentration). The pellet containing D protein was suspended in 50 mM Gly-NaOH buffer containing 2 mM EDTA and 5% glycerol at pH 10.5 using a Turrax homogenizer. The resulting suspension after addition of Triton X-100 (1% final concentration) was stirred for about 1 hour in a 4°C cold room, then centrifuged. The pellet containing D protein was dissolved in 8 M urea, 50 mM Tris at pH 8.0, overnight at 4°C. The supernatant contained the D protein.

This supernatant, after the addition of DTT (to 50 mM final concentration), was stirred under ambient conditions for about 1 hour then loaded onto a DEAE Fast Flow Sepharose [Pharmacia] column equilibrated with 8M urea and 50 mM Tris buffer at pH 8. D protein was eluted with a 0 to 0.3 M NaCl gradient (over five column volumes or greater) in equilibration buffer.

Fractions containing D protein were concentrated on a Minisette tangential flow apparatus [Pharmacia], treated with 10 mg SDS per mg of protein, DTT [Sigma] (to a final concentration of 50 mM) and stirred at ambient temperature for about 1.5 hour.

The resulting solution was loaded onto a Superose-12 column [Pharmacia], equilibrated with 25 mM Tris-Glycine buffer at pH 8,6, containing 1% SDS. The D protein was eluted at its predicted monomer MW in an isocratic gradient of equilibration buffer. Fractions containing D protein of adequate purity were pooled, concentrated,

treated with 10 mg SDS per mg of protein and DTT (to 50 mM final concentration) and then chromatographed on a Superose-12 column under the same conditions described immediately above.

Fractions containing D protein of the highest purity were pooled, concentrated and loaded onto a G25 Sephadex fine column [Pharmacia], equilibrated with 50 mM Tris buffer at pH 8.0 containing 8 M urea. D protein was eluted isocratically with equilibration buffer and fractions containing D protein free of SDS were pooled and concentrated. This highly purified sample of D protein was then concentrated, dialyzed against 20 mM Tris, 1 mM EDTA, pH 8.0 and sterile filtered.

Example 14 - Evaluation of Flu D Vaccine Compositions

Detailed descriptions of the D protein, and methods used for in vitro T cell assays and protection studies are described in S. B. Dillon et al, Vaccine, 10:309 (1992), incorporated by reference herein.

CTL and proliferation assays were performed as described previously [S. B. Dillon et al, cited above]. IL-2 was measured on an IL-2 dependent CTL line (CTLL) and IFNγ was measured by a commercial ELISA kit.

Vaccine compositions according to this invention containing superose purified D protein (Example 13) in aluminum hydroxide adjuvant, with or without 3D-MPL, were prepared as follows: 3D-MPL (RIBI Immunochemical, Hamilton, MT) was reconstituted in sterile water for injection to a working concentration of 1 mg/ml. This stock solution was sonicated for 30 minutes, and subsequent dilutions, made in 5% dextrose, were sonicated for an additional 10 minutes prior to addition to mixtures of the D protein in aluminum hydroxide, prepared as described previously [Dillon et al, Vaccine, 10(5):309-318 (1992)].

The antigen doses were titrated from 0.01, 0.1, 1.0, 10, 20, to 100 µg/dose of injection; and the 3D-MPL doses were titrated from 0.025, 0.25, 2.5, 25, to 50 µg/dose of injection. The ratio of 3D-MPL:antigen was maintained at

2.5:1 (w/w) for all antigen doses except the 100 µg dose, as shown in Table 5 below. Aluminum hydroxide was 100 µg per dose in all cases. A control vaccine composition contained 20 µg antigen mixed with CFA, as described in Dillon et al, Vaccine, 10(5):309-318 (1992). The final injection volumes were 0.2 ml per mouse.

For these protection studies, mice ($CB6F_1$; $H\text{-}2^{dxb}$) were injected subcutaneously with a selected vaccine dose, at weeks 0 and 3, and challenged intranasally (under metofane anesthesia) at week 7 with 2 to 5 LD_{50} doses of A/Puerto Rico/8/34 [A/PR/8/34 (influenza type A, H1N1)] virus.

Detailed descriptions of the methods used for protection studies are described in S. Dillon et al, Vaccine, 10(5): 309-318 (1992), incorporated by reference herein. Percent survival at day 21 post-challenge was compared between groups by the Fischers exact probability test.

Table 5

Antigen Dose (µg)	3D-MPL Dose (µg)	%Survival (Day 21)
100	50	27
100	0	53[+]
20	50	86[+,++]
20	0	40[**]
10	25	67[+]
10	0	40[**]
1.0	2.5	80[+,++]
1.0	0	7
0.1	0.25	20
0.1	0	20
0.01	0.025	20
0.01	0	13
0.0	50	13
0.0	0	0

[+] $p \leq 0.003$ vs adjuvant control.
[**] $p \leq 0.01$ vs adjuvant control.
[++] $p \leq 0.01$ vs aluminum formulation at same antigen dose.

The results of Table 5 show that the minimum dose of flu D antigen required for significant protection from lethal challenge was reduced from 10 µg (with aluminum only) to 1 µg when 3D-MPL was included in the vaccine composition. Also, the percentage of mice surviving challenge was increased when the 20 µg ($p \leq 0.01$), 10 µg ($p \geq 0.05$; NS) and 1 µg ($p \leq 0.01$) antigen doses were administered with 3D-MPL as well as aluminum vs. aluminum only in the vaccine composition. Survival in a CFA control group at 20 µg antigen was 73% in this

experiment.

Example 15 - Evaluation of Flu D Vaccine Compositions

This experiment was performed essentially as described in Example 14 above except that the dose of 3D-MPL was titrated and administered either alone, with the minimal effective dose of flu D antigen (1 µg) and the aluminum adjuvant (100 µg), or with flu D antigen (10µg) and the aluminum adjuvant (100 µg). The results reported in Table 6 identify 2 µg 3D-MPL as the minimal effective dose.

Table 6

Antigen Dose (µg)	3D-MPL Dose (µg)	% Survival (Day 21)
10	10	80^{+}
10	2.0	73^{+}
10	0.4	53^{+}
10	0.0	53$^{\cdot}$
1	10	80$^{+,++}$
1	2.0	87$^{+,++}$
1	0.4	33$^{\cdot}$
1	0.0	33
0	10	0
0	2.0	0
0	0.4	0
0	0.0	7

$^{+}$ $p \leq 0.001$ vs adjuvant control.

$^{\cdot}$ $p \leq 0.02$ vs adjuvant control.

$^{++}$ $p \leq 0.01$ vs aluminum formulation at same antigen dose.

Example 16 - Evaluation of Flu D Vaccine Compositions

In this experiment, the potency of liposomes plus 3D-MPL was compared to liposomes (Lipo) without 3D-MPL

[Ribi Immunochem], and to Al(OH), or CFA, by determining the level of protection seen after challenge with 2, 10 or 50 LD_{50} doses of virus. The vaccine used was the same as in Table 9 below.

Mice were injected at weeks 0 and 3 and challenged with A/PR/8/34 at week 7. Survival is shown in Table 7 below through day 21.

Table 7

Antigen	Dose (µg)	Adjuvant	% Survival Challenge Dose (LD_{50}) 2	10	50
D protein	50	Lipo	53	53[b]	7
	0	Lipo	27	0	0
D protein	50	Lipo/3D-MPL[a]	100[b]	73[b,c]	47[b,c]
	0	Lipo/3D-MPL	13	0	0
D protein	50	Al^{3+}	86[b]	20	13
	0	Al^{3+}	7	13	0
D protein	50	CFA	86[b]	80[b,c]	13
	0	CFA	33	0	0

[a] The amount of 3D-MPL per liposome was not determined.
[b] $p \leq 0.003$ vs. adjuvant control.
[c] $p \leq 0.003$ vs. aluminum hydroxide formulation at same antigen dose.

The results showed that significant protection against a 50 LD_{50} challenge was achieved with the liposome/3D-MPL formulation but not with any of the other adjuvant formulations tested (Table 7). Significant protection was achieved against the 2 LD_{50} challenge with all formulations, and all formulations except aluminum were protective against the 10 LD_{50} challenge dose. Therefore, the D protein in the 3D-MPL/liposome formulation constitutes a vaccine with even greater

potency than the D protein in CFA, which is the classic adjuvant for supporting T cell response, but is not suitable for use in humans.

Example 17 - Evaluation of Flu D Vaccine Compositions

In this experiment, a vaccine composition of the invention containing flu D protein, 3D-MPL, and liposomes was evaluated in comparison to a vaccine composition containing flu D, 3D-MPL and aluminum adjuvant. The actual amount of 3D-MPL incorporated into liposomes was measured.

The flu D protein (superose purified) in 25 mM Tris/1 mM EDTA, pH 8, at a total protein concentration of 2.49 mg/mL was dialyzed against 20 mM ammonium bicarbonate pH 8 and lyophilized to a powder in 20 mg aliquots.

Egg phosphatides (Asahi Type 5) and oleic acid (Sigma) were admixed at approximately a 1:1.2 molar ratio. L-arginine (free base), 0.38 M in water was added (1:1 molar ratio phosphatide:arginine) to the phosphatide/fatty acid mixture and mixed until a smooth and homogeneous gel is formed. This gel was the pre-liposomal gel.

The lyophilized protein was combined with the pre-liposomal gel (0.06-0.08 protein/gel (w/w) ratio) and mixed at room temperature until a homogeneous paste is formed. Monophosphoryl lipid A (Ribi/Immunochem) was added at a ratio of 1 µmole of lipid A to 66 µmole of phosphatides. This was a transparent colorless system composed of nanoparticulate (submicron in diameter) structures of aggregated 3D-MPL monomers. The resulting liposome suspension was further diluted with 25 mM Tris/1 mM EDTA, pH 8.0 buffer and homogenized. The liposome preparation was then serially centrifuged (3x) to remove unincorporated protein and 3D-MPL. Each time the pellet was resuspended with the Tris/EDTA buffer. The final liposome suspension was analyzed and kept stored under N_2 at 5°C while not in use.

The liposomes were subsequently assayed to determine

protein content by the modified Lowery colorimetric assay, the phospholipid concentration by the Barlett assay [Bartlett, G. R., J. Biol. Chem., 234:466-468 (1959)] and liposome size by photon correlation spectroscopy [Malvern Model 4700]. All assays were performed by standard techniques. Liposomes were not analyzed for lipid A content, however, it is likely that all of the lipid A remains in the final liposome fraction.

In the following Table 8, D protein LA and control LA-I was prepared by incorporating solid lipid A into the lipid phase. Control LA-II was prepared by hydrating with a solution of lipid A for incorporation.

Table 8

Liposome Sample	Phospholipid (µmole/mL)	Protein (mg/mL)	3D-MPL (µg/mL)	Diameter Z-Avg Mean (nm)
Control	4.37	not detected	not determined	200.8
Control+ 3D-MPL	7.87	0.02	not determined	201.3
Flu D	21.8	4.89	not detected	487.0
FluD+ 3D-MPL	14.8	4.06	270	502.4

Mice were immunized s.c. with 0.2 ml of the antigen/carrier/3D-MPL formulation at weeks 0 and 3, and challenged intranasally on week 7 with 5 LD_{50} of A/PR/8/34 virus. The "carrier" was either aluminum hydroxide (Al) or liposomes (Lipo). Survival was monitored for 21 days. These results are illustrated in Table 9 below. Unless

otherwise indicated, the p Value is measure vs. a 3D-MPL control.

Table 9

Antigen Dose	Carrier	Dose 3D-MPL	% Survival	p Value
1.0 µg	Al	-	33	0.084
1.0 µg		2.5 µg	80	0.000/0.001[a]
0.1 µg	Al	-	33	0.084
0.1 µg		2.5 µg	93	0.000/0.001[a]
0 µg	Al	2.5 µg	7	-
1.0 µg	Lipo	-	73	0.001
1.0 µg		0.05 µg	80	0.000
0.1 µg	Lipo	-	46	0.054
0.1 µg		0.005 µg	87	0.000/0.020[a]
0 µg	Lipo	0.05 µg	13	-

[a] p value vs. homologous dose without 3D-MPL.

This data indicates that by using liposomes as a carrier, the required dose of 3D-MPL (0.005 - 0.05 µg) can be dramatically reduced vs. the amount required with aluminum (about 2 µg). (See also Table 6 above).

Example 18 - Evaluation of Vaccine Composition

CB6F$_1$ mice (12 per group) were injected sc at weeks 0 and 3 with a vaccine composition comprising 100 µg flu D in aluminum (100 µg) plus 3D-MPL (10 µg). At week 7, the mice were challenged with 3-5 LD_{50} doses of the viruses shown in Fig. 1. Survival was monitored for 21 days post-challenge.

The results of Fig. 1 demonstrate that the antigenic specificity of the vaccine was equivalent to that

demonstrated earlier with the Al^{+3} adjuvant [S. B. Dillon et al, cited above] (i.e., cross-protective for both H1 and H2 subtypes, but lack of efficacy for H3 or Type B). Survival was also greater in the vaccinated group challenged with the heterologous H1N1 virus, A/Tai/86, although the results were not significant, $p < 0.09$. (See Fig. 1).

Example 19 - Evaluation of Flu D Vaccine Composition

$CB6F_1$ mice were injected subcutaneously (sc) with a vaccine composition comprising 1 µg D protein in aluminum (100 µg) plus 3D-MPL (2.5 µg) or with aluminum only at 0 and 3 weeks. Eighteen mice were challenged at week 7 with 5 LD_{50} doses of A/PR/8/34 virus. Five mice were sacrificed at day 7 post-challenge for lung titers. (Death in control groups at day 7 was 60% for Al/3D-MPL and 40% for CFA). Spleens were removed from 2 mice per group pre-challenge and 3 mice per group at day 6 post-challenge for proliferation and cytokine assays (Table 15 below). Table 10 reports survival (n=10/group) and lung virus clearance (n=5/group) after 5 LD_{50} challenge. Lung virus titer was recorded on day 7 in the fourth column of Table 10.

The results in Table 10 show that the reduction in lung virus titers in mice given a 5 LD_{50} challenge was of greater magnitude with 3D-MPL vs. CFA (2.4 log_{10} vs. 0.9 log_{10}), although survival was equivalent in these groups.

Table 10

Antigen (µg)	Adjuvant	Survival	log_{10} $TCID_{50}$/lung
1.0	Al/3D-MPL	86%*	4.41 ± 0.83+
0	Al/3D-MPL	0%	6.80 ± 0.45
1.0	Al	33%	6.39 ± 0.41+
0	Al	0%	7.39 ± 0.41
1.0	CFA	86%**	6.00 ± 0.50++
0	CFA	26%	6.90 ± 0.72

* $p \leq 0.001$ vs. adjuvant control group.
** $p \leq 0.01$ vs. adjuvant control group.
\+ $p \leq 0.003$ vs. adjuvant control group.
++ $p \leq 0.03$ vs. adjuvant control group.

Example 20 - Evaluation of Flu D Vaccine Composition

To determine if splenic T cell proliferation, γIFN production were correlated with protection, these responses were monitored in the spleens of mice immunized with 1 µg of D protein in Al vs Al/3D-MPL (2.5 ng 3D-MPL) adjuvants (from the study shown in Table 10) pre- and post-challenge. At week 7, two mice were sacrificed 6 days after virus challenge. Spleens were co-cultured with D protein and pulsed with ^{3}H-thymidine on day 3. Culture supernatants were harvested 8 hours later.

Overall, splenic proliferative responses were similar pre- vs. post-challenge for groups vaccinated with either D protein in the Al or Al/3D-MPL formulation (maximal cpm in unstimulated cultures equalled 2750 cpm) (Fig. 3). The magnitude of the proliferative response was also similar for either adjuvant group with the maximum stimulation index equally 3.0 or lower (Fig. 2). Pre-challenge gamma-interferon levels in antigen-stimulated culture supernatants were only modestly (about 2 fold) elevated and the two adjuvant groups were

equivalent as shown in Table 11.

However, in contrast, gamma-interferon production was increased greater than 7 fold in antigen-stimulated cultures from the Al/3D-MPL group post-challenge; whereas post-challenge levels did not rise in the group vaccinated with Al^{+3} only (Table 11). These results thus provide evidence that interferon-gamma production is correlated with reduced lung titers and survival post-challenge, and further show that T cell proliferation per se does not reflect the difference between the two adjuvant groups.

Table 11

	Injection			ng/ml IFN-gamma		
Antigen	Dose		Adjuvant	µg/ml in vitro	Pre-challnge	Post-challnge
D	1 µg		Al^{+3}/3D-MPL	0	<0.9	<0.9
				1	1.0	>7.0
				10	1.8	>7.0
-	-		Al^{+3}/3D-MPL	0	<0.9	<0.9
				1	<0.9	1.0
				10	0.94	<0.9
D	1 µg		Al	0	<0.9	<0.9
				1	1.9	1.0
				10	1.9	1.0
-	-		Al	0	<0.9	1.2
				1	<0.9	1.7
				10	1.1	1.0

Example 21 - Evaluation of Flu D Vaccine Composition

To further investigate a role for $CD4^+$ cells, antigen-specific proliferation and cytokine production were compared in mice vaccinated with the vaccine compositions containing flu D and aluminum adjuvant (Al) vs. vaccine compositions containing flu D and Al/3D-MPL formulations.

Lymph nodes from mice given a single injection of 1, 5 or 20 µg D protein in Al/3D-MPL (ratio of 3D-MPL:antigen at 2.5:1 w/w) or Al^+ adjuvant (100 µg) 7 days earlier were cultured with 0-30 µg/ml purified D protein.

The results are illustrated in Figs. 3 and 4. Proliferation was clearly increased in the groups that received Al/3D-MPL adjuvant, and the difference was greatest at the lowest *in vivo* antigen dose (1 µg). In Fig. 3 maximal cpm in unstimulated cultures equalled 1368 cpm. In Fig. 4 maximal cpm in unstimulated cultures equalled 1600 cpm; and maximal cpm of CTLL (an IL-2 dependent CTL line) cells cultured with supernatants from unstimulated cultures equalled 195 cpm). Peak (48 hours) IL-2 activity was greater in the groups vaccinated with the Al/3D-MPL formulations, although the levels were generally low in all cultures (Fig. 4).

Table 12 provides the results of the analysis of interferon-gamma in supernatants from the same antigen-stimulated cultures. Interferon levels in supernatants from adjuvant control cultures were all <1 ng/ml. Interferon-gamma was measured with Gibco-BRL ELISA kit. This cytokine, which was highest at day 4 of culture, was up to 5-fold greater in the Al/3D-MPL group.

Table 12

µg antigen (in vivo)	Adjuvant (µg/mL)	antigen in vitro	IFN-gamma (ng/ml) day 2	day 3	day 4
1.0	Al[+]	0	<1	<1	<1
		1	<1	<1	<1
		10	<1	<1	1.1
1.0	Al/3D-MPL	0	<1	<1	<1
		1	<1	<1	1.9
		10	1.1	2.9	4.9

The results from Table 11 and 12 collectively support a role for interferon gamma in the mechanism of action of MPL adjuvant.

Example 22 - Evaluation of Flu D Vaccine Composition

Since the above examples indicate that 3D-MPL improves potency of the vaccine, a study was done to determine if the booster injection was necessary for protection. Mice were vaccinated with a single sc injection of 50 µg D and challenged 7 weeks later with 2 LD_{50} A/PR/8/34. Alternatively, a second group was given a booster injection with the same antigen dose at 3 weeks. The 3D-MPL dose was 125 µg. The flu D was adjuvanted with either aluminum hydroxide, aluminum plus 3D-MPL, or CFA. Controls for each adjuvant were performed.

The results in Table 13 below (shown in Example 23) show that the incorporation of 3D-MPL (125 µg) into a vaccine formulation with 50 µg of antigen significantly increased survival in mice given either one or two injections when compared to the same dose of the vaccine protein adsorbed to aluminum. Again, survival in the Al/MPL group was comparable to that seen in the CFA.

Example 23 - Cytotoxic T Lymphocyte Assays

Detailed descriptions of the methods used for *in vitro* T cell assays are described in S. Dillon et al, cited above. Assays to detect memory CTL were performed after secondary *in vitro* stimulation with virus as described previously [See, F. Ennis et al, Lancet, II:887-891 (1981); A. Yamada et al, J. Exp. Med., 162:663-674 (1985); and K. Kuwono et al, J. Exp. Med., 169:1361-1371 (1989)]. Briefly, spleen or lymph node cells from immune or control mice were cultured at a ratio of 6:1 with influenza virus-infected, syngeneic spleen cells for 5 days. Culture medium was RPMI 1640 supplemented with 10% fetal calf serum [Hyclone Laboratories, Logan, UT], 2 mM glutamine, 5 x 10^{-5} M 2-ME, 10 mM HEPES, penicillin and streptomycin.

The spleens from mice given one or two sc injections of 50 µg D protein (0 and 3 weeks), were removed at week seven and restimulated *in vitro* as described above. One lytic unit (LU_{35}) is defined as the number of effector cells in the chromium release assay required for 35% lysis (determined by regression analysis). Table 13 reports the results of H1N1-specific CTL activity in these spleens of mice immunized with SK&F 106160 (D Protein) in aluminum hydroxide and 3D-MPL.

Table 13

LU_{35} per culture

	Control Targets		A/PR/8/34 Targets		A/Taiwan/86 Targets		Percent Survival	
# Injections:	1	2	1	2	1	2	1	2
Formulation								
D/Al^{+3}*	<1	19	53	56	51	69	7	33
D/Al^{+3}/3D-MPL*	<1	<1	41	43	64	53	$40^{+,++}$	$73^{+,++}$
D/CFA*	<1	<1	129	88	98	131	$53^{+,++}$	$93^{+,++}$
Al^{+3}	<1	<1	<1	<1	35	35	N.D.	7
Al^{+3}/3D-MPL	18	18	<1	<1	29	29	N.D.	0
CFA	<1	<1	<1	<1	<1	<1	N.D.	0

* = Antigenic dose is 50 µg flu D protein.
+ = $p < 0.05$ vs. adjuvant control group
++ = $p < 0.05$ vs. Al^{3+} adjuvant group

In the experiments of this example, 3D-MPL did not potentiate class I restricted memory CD8+ CTL relative to the response generated with aluminum hydroxide (indicated as Al^{+3}) as reported in Table 13. In addition, the results in Table 13 show that splenic CTL were comparable in mice given 1 vs. 2 sc injections, whereas protection was clearly improved in mice given a second injection.

Therefore it appears that a mechanism other than CD8+ CTL must be responsible for the improved immunity seen when 3D-MPL is included in the vaccine composition containing the antigenic peptide and aluminum adjuvant, or when mice are given a booster injection.

Example 24 - Depletion Studies

To further determine which T cell subset is best correlated with the activity of the 3D-MPL adjuvant, depletion studies were done with anti-CD-4 or anti-CD-8 monoclonal antibodies (Mabs). An initial study was performed using a post-vaccination protocol for T cell subset depletion as follows. Mice (15/group) were immunized with two subcutaneous injections of 50 ug flu D (SK&F 106160) in Al/3D-MPL at 0 and 21 days, and challenged with 5 LD_{50} doses of A/PR/8/34 on day 42. Antibodies (300 µg/mouse) were administered ip post-vaccination on days 32, 33, 34, 39, 40 and 41 pre-challenge and on days 43, 48 and 53 post-challenge. In another study, mice were treated with Mabs to deplete T cell subsets prior to the first injection of vaccine (pre-vaccination protocol) on days -3, -2, -1. After the vaccination on day 0, mice were further treated with Mabs on days, 7, 14, 18, 19 and 20, and then boosted with vaccine on day 21. Mice were further treated with Mabs on days 28, 35, 39, 40 and 41 prior to virus challenge on day 42. Mice were further treated with Mabs post-challenge on days 43, 49 and 54. The results of these T cell subset depletion experiments are shown in Table 14 below. The results show that the effectiveness of vaccination was reduced when mice were depleted of either the $CD4^+$ or $CD8^+$ T cell subsets, the effect was more

pronounced when Mab treatment was initiated prior to vaccination. Therefore, these results definitively show that the mechanism of action of the flu D vaccine in Al/MPL formulation is T cell mediated, and both T cell subsets are required for activity.

Since production of gamma interferon correlated with protection (Table 11), a study was performed to determine the phenotype of T cell responsible for producing this cytokine in vaccinated mice. Mice were depleted of $CD4^+$ or $CD8^+$ T cell subsets by injecting anti-CD4 or anti-CD8 Mabs ip daily for 3 days (300 µg/injection). Four days after the last Mab injection, mice were immunized with 20 µg flu D protein in the aluminum hydroxide (100 µg) and 3D-MPL (20 µg) formulation, and lymph nodes were removed 7 days later. The lymph node cells were restimulated *in vitro* with 0, 1 or 10 µg/ml D protein, and supernatants collected on days 1-4 for interferon and IL-2 assays. The results in Fig. 5 show that IL-2 production was completely eliminated by anti-CD4 treatment, but was also partially reduced in anti-CD-8 treated mice (Fig. 5B). Peak IFNγ production (day 4 supernatants) was reduced by approximately 50% in anti-CD4 or anti-CD8 treated mice (Fig. 5C). Therefore, both $CD4^+$ and $CD8^+$ T cell subsets produced IL-2 and IFNγ.

Table 14

Antigen (μg)	3D-MPL (μg)	Mab Treatment	% Survival (Mabs Post-Vaccination)	% Survival (Mabs Pre-Vaccination)
50	10	none	70*	80*
50	10	anti-CD4	30	20+
50	10	anti-CD8	50**	0+
50	10	anti-CD4+ anti-CD8	30	10+
0	10	none	0	0

* $p \leq 0.002$ vs. adjuvant control (group 5)
** $p \leq 0.02$ vs. adjuvant control (group 5)
+ $p \leq 0.01$ vs. untreated control (group 1)

The examples presented above demonstrate that a recombinant influenza H1N1 vaccine formulated with aluminum plus 3D-MPL facilitated virus clearance and survival, and reduced the antigen dose required for significant protection against lethal challenge, reaching a level of potency equivalent to that seen with CFA. The data collected to date provides evidence that the mechanism by which 3D-MPL acts to potentiate the activity of this recombinant influenza vaccine appears to be via the selective potentiation of $CD4^+$ T cell responses, and may be restricted to TH1-type cells which produce IL-2 and IFNγ.

Example 25 - Preparation of Split Virus

Split viruses, such as those produced by Sachsisches Serumwerkl GmbH (SSW) (Dresden, Germany), may be prepared

by well known methods, such as those documented in, for example, European Pharmacopoeia PA/PH/Exp 3 (1992); DAB 10 "Vaccines Influenzae ex virorum fragmentis praeparatum" and the World Health Organization draft revised requirements for Influenza vaccines (Inactivated) (1990). Such split viruses are prepared using one or more influenza virus strains recommended by WHO and the EEC, such as A/Singapore/6/86, A/Beijing/32/92 and B/Panama/45/90. These strains can change depending on the strains popular in a particular year.

As described in more detail elsewhere, influenza viruses are obtained from embryonated hens' eggs inoculated with seed lot material. These virus suspensions are partially purified and concentrated. The concentrated virus suspension is treated with a detergent, sodium desoxylcholate, to disrupt (or "split") the virus particles. Following the removal of viral phospholipids during the splitting process, the reactogenicity potential is greatly reduced. The split virus suspension is completely inactivated by the combined effect of the detergent and formaldehyde.

More specifically, the process for producing a split virus of this invention is as follows.

A. Preparation of monovalent whole virus inoculum

On the day of inoculation of embryonated eggs a fresh inoculum is prepared by mixing the influenza working seed lot with a phosphate buffer containing gentamycin sulphate at 0.5 mg/ml and hydrocortisone at 25 µg/ml. The virus inoculum is kept at 2-8°C.

Nine to eleven day old embryonated eggs are used for virus replication. The eggs are incubated at the farms before arrival at the manufacturing plant and enter into the production rooms after decontamination of the shells. The eggs are inoculated with 0.2 ml of the virus inoculum on an automatic egg inoculation apparatus. The inoculum is injected at a pressure of ± 0.03 MPa. The inoculated eggs are incubated at the appropriate temperature (virus strain-dependent) for 50 to 96 hours. At the end of the incubation period, the embryos are killed by cooling the

eggs and storage for 12-60 hours at 2-8°C.

The allantoic fluid from the chilled embryonated eggs is harvested by appropriate egg harvesting machines. Usually, 8 to 10 ml of crude allantoic fluid can be collected per egg. To the crude monovalent virus bulk is add 0.100 mg/ml thiomersal.

The harvested allantoic fluid is clarified by flow through centrifugation at a volume of 100-200 L/hour and a centrifugal force of 10-17,000 g. This preclarified liquid can be further clarified on a 6-8 µm membrane filter.

To obtain a $CaHPO_4$ gel in the clarified virus pool, 0.5 M Na_2HPO_4 and 0.5 M $CaCl_2$ solutions are added to reach a final concentration of $CaHPO_4$ of 1.5 g to 3.5 g $CaHPO_4$/liter depending on the virus strain. After sedimentation for at least 10 hours, the supernatant is removed and the sediment containing the Influenza virus is resolubilized by addition of a 0.26 M EDTA solution. The concentration of EDTA varies between 4.5 and 10 g/liter of the original harvest volume.

The resuspended sediment is filtered on a 6 to 8 µm filter membrane.

The Influenza virus is concentrated by isopycnic centrifugation in a linear sucrose gradient (0-55%) at 90,000 g. The flow through volume is from 8-12 liters/hour. At the end of the centrifugation, the content of the rotor is recovered by four different fractions (the sucrose is measured in a refractometer):

- fraction 1 55-52% sucrose
- fraction 2 52-38% sucrose
- fraction 3 38-20% sucrose
- fraction 4 20-0% sucrose

For further vaccine preparation, only fractions 2 and 3 are used. Fraction 3 is diluted in order to reduce the sucrose content to approximately 6%. The Influenza virus present in this diluted fraction is pelleted at 53,000 g to remove soluble contaminants. The pellet is resuspended and thoroughly mixed to obtain a homogenous suspension. Fraction 2 and the resuspended pellet of

fraction 3 are pooled and phosphate buffer is added to obtain a volume of 30 liters. At this stage, the product is called "monovalent whole virus concentrate".

B. Split virus monovalent bulk

The selected Influenza virus, preferably the monovalent whole virus concentrate of Part A above, is disrupted by centrifugation at 70,000 g through a Nadoc linear sucrose gradient of 0-55% containing a linear distribution of sodiumdesoxycholate from 1,2-1,5%. Tween 80 is present at 0.1% in the gradient. The virus concentrate is pumped at the rate of 5 liters/hour. At the end of the centrifugation, the content of the rotor is collected in 3 different fractions:

Fraction 1: 55-40% sucrose
Fraction 2: 40-13% sucrose
Fraction 3: 13-0% sucrose

The haemagglutinin is concentrated in fraction 2. Phosphate buffer containing thiomersal at 0.01% and Tween 80 at 0.01% is added to dilute the fraction four times (± 5 liters).

The diluted fraction 2 is filtered on filter membranes ending with a 0.2 µm membrane. At the end of the filtration, the filters are washed with phosphate buffer containing 0.01% thiomersal and 0.01% Tween 80. As a result, the final volume of the filtered fraction 2 is 5 times the original fraction volume. Depending on the virus strain, a brief sonication of the split virus material can be introduced to facilitate the sterile filtration.

The filtered monovalent split material is incubated at 22 ± 2°C for at least 84 hours to allow inactivation of viruses and mycoplasma by the effect of sodium desoxycholate. After this incubation time, phosphate buffer containing 0.01% thiomersal and 0.01% Tween 80 is added in order to bring the total protein content down to maximum of 250 µg/ml. Formaldehyde is added at the rate of 50 µg/ml and the inactivation takes place at 4°C ± 2°C for at least 72 hours.

The inactivated split virus material is

ultrafiltered on membranes having a mean pore size of 20,000 daltons. During ultrafiltration, the content of formaldehyde, NaDOC and saccharose is greatly reduced. The volume remains constant during ultrafiltration (diafiltration) by adding phosphate buffer containing 0.01% thiomersal and 0.01% Tween 80.

The ultrafiltered split material is filtered on membranes ending with a 0.2 µm, depending on the virus strain the last filtration membrane can be 0.8 µm. At this stage, the product is called: "monovalent final bulk". The monovalent final bulk is stored at 2-8°C for a maximum of 18 months.

Example 26 - Split Virus Vaccine Composition

(a) Preparation of MPL with a particle size of 60 - 120 nm

Water for injection is injected in vials containing lyophilised 3 deacylated monophosphoryl lipid A (MPL) from Ribi Immunochem, Montana using a syringe to reach a concentration of 1 to 2 mg per ml. A preliminary suspension is obtained by mixing using a vortex. The content of the vials is then transferred into 25 ml Corex tubes with round bottoms (10 ml suspension per tube) and the suspension is sonicated using a water bath sonicator. When the suspension has become clear, the size of the particles is estimated using dynamic light scattering (Malvern Zetasizer 3). The treatment is continued until the size of the MPL particles is in the range 60 - 120 nm, and preferably below 100nm.

Suspensions can in some cases be stored at 4 degrees C without significant aggregation up to 5 months. Isotonic NaCl (0.15M) or isotonic NaCl plus 10mM phosphate induces a rapid aggregation (size >3-5µm).

(b) The split virus vaccine composition of this invention is prepared as follows.

A final bulk-buffer is prepared by adding to water for injection the concentrated salt solutions: NaCl 4 mg; Na_2HPO_4 0.52 mg; KH_2PO_4 0.19 mg; KCl 0.1 mg; and $MgCl_2$ 0.05

mg, and Thiomersal 50 µg. The resulting solution is stirred 15 minutes before further use.

Monovalent split virus bulk (15 µg HA) is then mixed with 3D-MPL 50 µg and the resulting mixture is stirred for about 1 hour at room (or ambient) temperature.

The buffer mixture and the virus bulk mixture are then mixed together. After 30 minutes stirring at room temperature, the pH is brought to 7.15 ± 0.1. The resulting final vaccine is stored at +2 - +8°C.

To prepare a multivalent split virus vaccine, such as a trivalent vaccine, monovalent split virus pools of the selected strains prepared as described above are mixed to constitute the final multivalent vaccine. The resulting pooled material is stirred for 30 minutes at room temperature (pH 7.15 ± 0.1). The resulting final vaccine may be stored at temperatures between about 2°C to about 8°C. The vaccine is a colourless, light opalescent aqueous suspension of purified split influenza virus.

For purposes of comparison in the tests of the following examples, a vaccine preparation was prepared without the adjuvant. For this control vaccine, a final monovalent bulk-buffer is prepared by adding to water for injection the concentrated salt solutions: NaCl 4 mg; Na_2HPO_4 0.52 mg; KH_2PO_4 0.19 mg; KCl 0.1 mg; $MgCl_2$ 0.05 mg; and Thiomersal 50 µg. The resulting solution is stirred 15 minutes before further use. The mixture is stirred for 15 minutes at room temperature. Monovalent bulk (15 µg HA) is added, followed by 30 minutes stirring at room temperature (pH 7.15 ± 0.1). The resulting final vaccine is stored at temperatures between +2°C - +8°C.

Two monovalent vaccine compositions of this invention and monovalent control vaccines were prepared for the following examples using the H1N1 strains, A/PR/8 and Singapore mixed with 3D-MPL 50µg (particle size < 100nm as obtained from Example 26 (a)).

Example 27 - Lethal Challenge in Mice

The immunogenicity of the monovalent vaccine formulation of this invention has been evaluated and

compared to the immunogenicity of the monovalent non-adjuvanted formula and to the adjuvant 3D-MPL without antigen in a lethal influenza challenge in mice.

For each vaccine, $CB6F_1$ mice (30 per group) were immunized subcutaneously with 2 injections given 3 weeks apart with a preparation that contains 5 µg of indicated strain ± 5 µg 3D-MPL. Seven weeks after the first injection, mice were intranasally challenged under metofane anaesthesia with 5 LD_{50} of A/PR/8. Survival and clinical signs of illness were monitored for 15 mice up to 3 weeks after challenge. Lung virus titers were determined by MDCK microassay [A. L. Frank et al, J. Clin. Microbiol., 12:426-432 (1980)] on the remaining animals (5 mice per group).

The results are reported in Table 15 below.

Table 15
Mice Lethal Challenge: Survival Rate and Lung Virus Titers

Vaccine Group	Survival (%)	Virus titer*		
		day 3	day 5	day 7
Alum + 3D-MPL	13	8.90±0.10	8.44±0.55	8.69±0.32
Singapore	80	8.10±0.17	7.15±0.23	3.09±0.10
Singapore + 3D-MPL	100#	<1	<1	<1
A/PR/8	100#	8.01±0.029	3.93±0.70	<1
A/PR/8 + 3D-MPL	100#	<1	<1	<1

* log TCID/lung (geometric mean ± S.E.)
\# no visible clinical symptoms

The two strains (A/PR/8 and Singapore) induce a certain level of protection. The survival rate for both groups was markedly higher than that of the control

preparation devoid of antigen (Alum + 3D-MPL), and lung virus clearance was faster. Not surprisingly, A/PR/8-vaccinated group (homologous with respect to challenge) performed better than the Singapore-vaccinated group (heterologous with respect to challenge). Furthermore, both strains supplemented with 3D-MPL induced 100% survival, practically no lung viral titer, and no clinical symptoms. Clearly the 3D-MPL adjuvanted vaccine of the invention induced superior protection against lower respiratory track infection and therefore serious clinical manifestations such as viral pneumonia. From this experiment it therefore appears that 3D-MPL adjuvantation improves homosubtypic and heterosubtypic activity of the split vaccine and is therefore anticipated to provide broader protection against antigenic shifts and drifts than unadjuvanted vaccine.

Example 28 - Non-Lethal Challenge in Mice

The protocol was the same as Example 25 above, except that mice were not anesthesized before intranasal challenge. Virus titration in the respiratory tract, virus neutralization assays and scanning electron microscopy of trachea were performed.

Viral neutralization titration against A/PR/8 virus was carried out on days 1, 5 and 9 post-challenge (Table 16).

Table 16
Viral Neutralization Assay

Vaccine Group	Neutralizing titer*		
	day 1	day 5	day 9
Alum + 3D-MPL	<1	<1	<1
Singapore	<1	<1	<1
Singapore +3D-MPL	<1	<1	<1
A/PR/8	1.6	2.3	2.2
A/PR/8 + 3D-MPL	3.5	3.6	3.3

* mean of $\log_{10}$ (5 mice)

Both A/PR/8 groups produced neutralizing antibodies, with a titer consistently higher in the presence of 3D-MPL. In contrast, the vaccination with Singapore did not induce neutralization antibodies, even with the addition of 3D-MPL. This indicated that the heterosubtypic activity observed previously with Singapore and 3D-MPL was not due to antibody and provides strong evidence that cell-mediated immunity was responsible.

Scanning Electron Micrographs (SEM) of trachea were performed on samples harvested on days 3 and 5. The tissues were evaluated for histophathological changes in the serious and cilliated epithelial cells lining the trachea particularly desqumation. These changes are indicative of the severity of an ongoing influenza infection or the recovery from infection (see Table 17 below).

Table 17

Non Lethal Challenge in Mice: SEM of Trachea

Vaccine Grp	Mean severity score*		Overall Score**
	day 1	day 5	
Alum + 3D-MPL	1.7	1.0	0
Singapore	2.0	1.7	2+
Singapore+MPL	2.4	2.4	3+
A/PR/8	2.0	2.0	2+
A/PR/8+3D-MPL	2.0	2.4	3+

* semi quantitative, done blind.
Regenerating: 1: severe lesion
2: partial lesion
3: normal tissue

** overall score, from 0 (no protection) to 4+ (complete protection)

The results of SEM suggest that vaccination with either A/PR/8 or Singapore strain alone gave protection; in both cases this positive effect is enhanced by 3D-MPL addition, thereby indicating that adjuvanted split vaccine may alter the progression of influenza disease.

Virus titers were determined in the nose, trachea and lungs by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group). Results are shown in Figs. 6A through 6F below. Not surprisingly, nasal titers were higher than tracheal or pulmonary titers. However, all the treatments assayed did not result in clear differences in nasal or tracheal titers. In contrast, lung titers were differentiated: A/PR/8 + 3D-MPL and Singapore + 3D-MPL titers were always lower or equal to the values of the antigen alone, or of 3D-MPL alone (Figs. 6A through 6F). This indicates that 3D-MPL

adjuvantation confers a better lung protection in mice.

Both sets of mice experiments (lethal and non lethal challenge) indicate that influenza vaccination with two subtypes of H_1N_1 strain (Singapore or A/PR/8) and subsequent homotypic challenge is improved by the incorporation of 3D-MPL as an adjuvant according to this invention.

Example 29 - Immunogenicity of the Vaccine

For each vaccine, $CB6F_1$ mice (15 per group) were immunized subcutaneously with 2 injections given 3 weeks apart of a preparation that contains one tenth of a human dose of conventional unadjuvanted Singapore monovalent split vaccine or a vaccine composition of this invention containing the Singapore strain with 3D-MPL. A human dose is defined as a 0.5 ml injection containing 15 µg HA of each viral strain. The formulation used herein contained a hemaglutinin (HA) to 3D-MPL ratio of 1.5 µg HA per 5 µg 3D-MPL to 5 µg HA per 5 µg 3D-MPL. A control group received only 3D-MPL (5 µg). Three weeks after the second injection, mice were bled and sera antibodies were individually assayed by inhibition of haemagglutination. Titers were calculated against a calibrated reference. Mice were considered responders if they have antibody titers greater than the cut-off value.

The results are reported in Table 18. Both types of formulations induce anti-haemagglutination antibodies in all animals, thus the seroresponse rate is maximal. However, the immune response is significantly enhanced by the vaccine formulation of the present invention containing 3D-MPL, as indicated by a geometric mean titer more than 5 times higher than that of the vaccine containing the Singapore strain alone.

Table 18

Vaccine Group	Individual titers*	Geometric mean titer	Seroresponse
3D-MPL (5 µg)	<25; <25; <25; <25; <25; <25; <25; <25; <25; <25; <25; <25; <25; <25; <25	<25	0/15
Singapore (1.5 µg HA)	100; 200; 200 75; 200; 100 25 800 300 50 200 200 600 200 50	150	15/15
Singapore (1.5 µg HA) + 3D-MPL 5 µg	1200 600 400 400 1600 800 200 1600 1600 400 400 3200 400 3200 1200	828	15/15

* cut-off value is lower than 25

This experiment indicates that antibody response in mice is improved by the use of the 3D-MPL adjuvanted vaccine of this invention.

Example 30 - Hypersensitivity Study in Guinea Pig

A hypersensitivity study was conducted in guinea pigs to determine whether the addition of 3D-MPL (particle size < 100 nm, Example 26) to a trivalent split vaccine modifies hypersensitivity. The trivalent vaccine, designated Trivalent Influsplit, was prepared as described in Example 25 and contained an H1N1 strain Singapore/6/86, an H3N2 strain Beijing/353/84 and a Type B strain, B/Yamaghta/16/88.

The sensitizing agent [allantoic fluid 0.5 or 2.5 mg ± 3D-MPL 50 µg; one human dose (0.5 ml injection containing 15 µg HA for each of these strains of influenza virus) Trivalent Influsplit ± 3D-MPL 50 µg] was given intraperitoneally by six injections, at days 0, 3, 5, 7, 10, and 12. Guinea pigs were allowed to rest for 4 weeks and then challenged intravenously, under anesthesia, with the challenging agent (allantoic fluid

0.45 mg; one human dose Trivalent Influsplit ± 3D-MPL 50 µg). The animals were observed 30 minutes after challenge, and again after 2 or 3 hours. The observed symptoms (scratching, breathing problems, convulsions, death) were recorded. Where no symptoms arose from the first challenge, the animals were re-challenged with allantoic fluid (1.3 mg) 24 hours later.

The results of this assay are shown in Tables 19A and 19B below.

Table 19A - Hypersensitivity Studies of Influsplit ± 3D-MPL

Group	Sensitization Agent	Challenge		
		Agent	Mortality	Other Findings
1 (negative ctrl)	none	allantoic fluid	0/5	none
2 (negative ctrl)	none	Influsplit	0/5	none
3 (negative ctrl)	none	Influsplit/ 3D-MPL	0/5	none
4 (positive ctrl allantoic	allantoic fluid	allantoic fluid	4/5	itching, dizziness convulsion
5 (test split)	Influsplit	allantoic fluid	0/5	none
6 (test split)	allantoic fluid	Influsplit	0/5	none
7 (positive ctrl allantoic/3D-MPL)	allantoic fluid 3D-MPL	allantoic fluid	4/5	itching, dizziness convulsion
8 (test split/ 3D-MPL)	- Influsplit - 3D-MPL	allantoic fluid	0/5	none
9 (test split/ 3D-MPL)	allantoic fluid	Influsplit/ 3D-MPL	0/5	none

Table 19B - Hypersensitivity Studies of Influsplit ± 3D-MPL

Group	Sensitization Agent	Re-challenge		
		Agent	Mortality	Other Findings
1 (negative ctrl)	none			
2 (negative ctrl)	none			
3 (negative ctrl)	none			
4 (positive ctrl allantoic	allantoic fluid	allantoic fluid	1/5	itching, dizziness convulsion
5 (test split)	Influsplit	allantoic fluid	0/5	none
6 (test split)	allantoic fluid	allantoic fluid	5/5	itching, dizziness convulsion
7 (positive ctrl allantoic/3D-MPL)	allantoic fluid 3D-MPL	allantoic fluid	1/1	itching, dizziness convulsion
8 (test split/ 3D-MPL)	- Influsplit - 3D-MPL	allantoic fluid	0/5	none
9 (test split/ 3D-MPL)	allantoic fluid	allantoic fluid	5/5	itching, dizziness convulsion

The absence of effect for groups 1 to 3 (negative controls) indicates that hypersensitivity requires prior intraperitoneal sensitization. The absence of hypersensitization in group 5 indicates that Influsplit cannot sensitize to allantoic fluid. A similar conclusion can be drawn from group 6 (sensitization to allantoic fluid and challenge with Influsplit). In fact, results from groups 5 and 6 also indicate that the vaccine does not contain residues of allantoic fluid proteins. The addition of 3D-MPL either to the sensitizing agent (groups 7 and 8) or to the challenging agent (group 9) did not change the response (compare group 7 vs 4; group 8 vs 5; group 9 vs 6).

This experiment demonstrated that the trivalent Influsplit vaccine given as sensitizing agent with or without 3D-MPL is not able to induce hypersensitivity. The trivalent Influsplit vaccine given as challenging agent with or without 3D-MPL is not able to trigger any hypersensivity reaction in animals previously hypersensitized with allantoic fluid; and within the experimental conditions, 3D-MPL does not have any noticeable effect on hypersensitivity reactions. Thus, the vaccine compositions of this invention are safe for administration to mammals.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

A/C

CLAIMS

1. A vaccine composition capable of eliciting an enhanced immune response to an influenza antigen comprising an effective amount of said influenza antigen and 3D-MPL.

2. The vaccine composition according to claim 1 wherein said influenza antigen is an antigenic polypeptide.

3. The vaccine composition according to claim 1 wherein said antigenic polypeptide is selected from the group consisting of D protein $NS1_{1-81}HA2_{65-222}$, $NS1_{1-81}HA2_{1-222}$, $HA2_{66-222}$, ΔM, ΔM+, A, C, C13, C13 short, ΔD, Cys-less D, $HA2_{66-222}$, and NS1H3HA2 constructs.

4. The vaccine composition according to claim 1 wherein said composition comprises at least one split influenza virus.

5. The vaccine composition according to claim 4 wherein said composition comprises three split viruses having reactivity against at least three strains of influenza virus.

6. The vaccine composition according to claim 4 wherein said strains are selected from H1N1, H3N2, H2N2, and Type B influenza strains.

7. The vaccine composition according to claim 6 wherein said split virus is derived from the group of influenza strains consisting of A/PR/8, A/Singapore, A/Udorn, A/Victoria, A/Texas, A/Beijing, A/Puerto Rico, B/Panama, B/Yamaghta, B/Lee/40, and B/Taiwan.

8. The vaccine composition according to claim 1 further comprising an aluminum-containing adjuvant.

9. The vaccine composition according to claim 8 wherein said adjuvant is aluminum hydroxide or aluminum phosphate.

10. The vaccine composition according to claim 9 wherein the influenza antigen is $NS1_{1-81}HA2_{65-222}$, the D protein.

11. The vaccine composition according to claim 10 consisting of between about 1 µg to about 1000 µg D protein and between about 1 µg to about 50 µg 3D-MPL.

12. The vaccine composition according to claim 11 wherein said amount of D protein is about 2 µg and said amount of 3D-MPL is about 20 µg.

13. The vaccine composition according to claim 3 wherein said antigen is the D protein, said composition comprising between about 50 µg to about 500 µg D protein, between about 10 µg to about 50 µg 3D-MPL, and between about 100 µg to about 500 µg aluminum adjuvant.

14. A vaccine composition according to any of claims 1 to 13 additionally comprising a liposome preparation, wherein said liposome preparation comprises liposome-forming material containing a long chain aliphatic or aromatic-based acid or amine; a hydrating agent of charge opposite to that of the acid or amine, which agent is present in a molar ratio of between 1:20 and 1:0.05 relative to the acid or amine; and water in an amount up to 300 moles relative to the solids present in the composition.

15. The vaccine composition according to claim 14 consisting of between about 50 µg to about 500 µg D protein, between about 10 µg to about 50 µg 3D-MPL, and between about 1 mg to about 10 mg liposome preparation.

16. The vaccine composition according to claim 14 wherein said antigen is flu D, consisting of between

about 50 µg to about 500 µg D protein, between about 10 µg to about 50 µg 3D-MPL, to about 1 mg to about 10 mg liposome preparation and between about 100 µg to about 500 µg aluminum adjuvant.

17. The vaccine composition according to any of claims 14 to 16 wherein the hydrating agent is an alpha amino acid having an omega substitution which is a carboxylate, amino, or guanido function or a pharmaceutically acceptable salt thereof, or a compound of the formula:

$$X-(CH_2)n-Y \quad I$$

wherein

X is $H_2N-C(NH)-NH-$, H_2N-, ZO_3S-, Z_2O_3P-, or ZO_2C- wherein Z is H or an inorganic or organic cation;

Y is $-CH(NH_2)-CO_2H$, $-NH_2$, $-NH-C(NH)-NH_2-COOH$, $CH(NH_2)SO_3Z$ or $ZH(NH_2)PO_3Z_2$ wherein Z is defined above; and

n is the integer 1-10; or

a pharmaceutically acceptable salt thereof and the acid or amine is an alkyl or alkenyl acid or amine of 10 to 20 carbon atoms.

18. The vaccine composition according to claim 17 wherein said hydrating agent is arginine, homoarginine, their N-acyl derivatives, gamma-aminobutyric acid, asparagine, lysine, ornithine, glutamic acid, aspartic acid or a compound of the formula:

$H_2NC(NH)-NH-(CH_2)_n-CH(NH_2)COOH$	II
$H_2N-(CH_2)_n-CH(NH_2)COOH$	III
$H_2N-(CH_2)_n-NH_2$	IV
$H_2NC(NH)-NH-(CH_2)_n-NH-CH(NH)-NH_2$	V
$HOOC-(CH_2)_n-CH(NH_2)COOH$	VI
$HOOC-(CH_2)_n-COOH$	VII
$HO_3S-(CH_2)_n-CH(NH_2)COOH$	VIII
$H_2O_3S-(CH_2)_n-CH(NH_2)COOH$	IX
$HO_3S-(CH_2)_n-CH(NH_2)SO_3H$	X, or
$H_2O_3S-(CH_2)_n-CH(NH_2)PO_3H_2$	XI

wherein n is 2-4, or a pharmaceutically acceptable salt thereof.

19. A vaccine composition according to any preceding claim wherein the particle size of the 3D-MPL does not exceed 120nm.

20. A vaccine composition according to claim 19 wherein said particle size is in the range 60-120nm.

21. A vaccine composition according to claim 19 or 20 wherein said particle size is below 100nm.

22. A method for enhancing an immune response to an influenza antigen comprising the step of internally administering to the mammal a vaccine composition of claim 1.

23. Use of a vaccine composition according to claim 1 in the manufacture of a medicament for enhancing an immune response to an influenza antigen.

24. A method for preparing a vaccine composition according to claim 1 which comprises admixing said antigen and 3D-MPL.

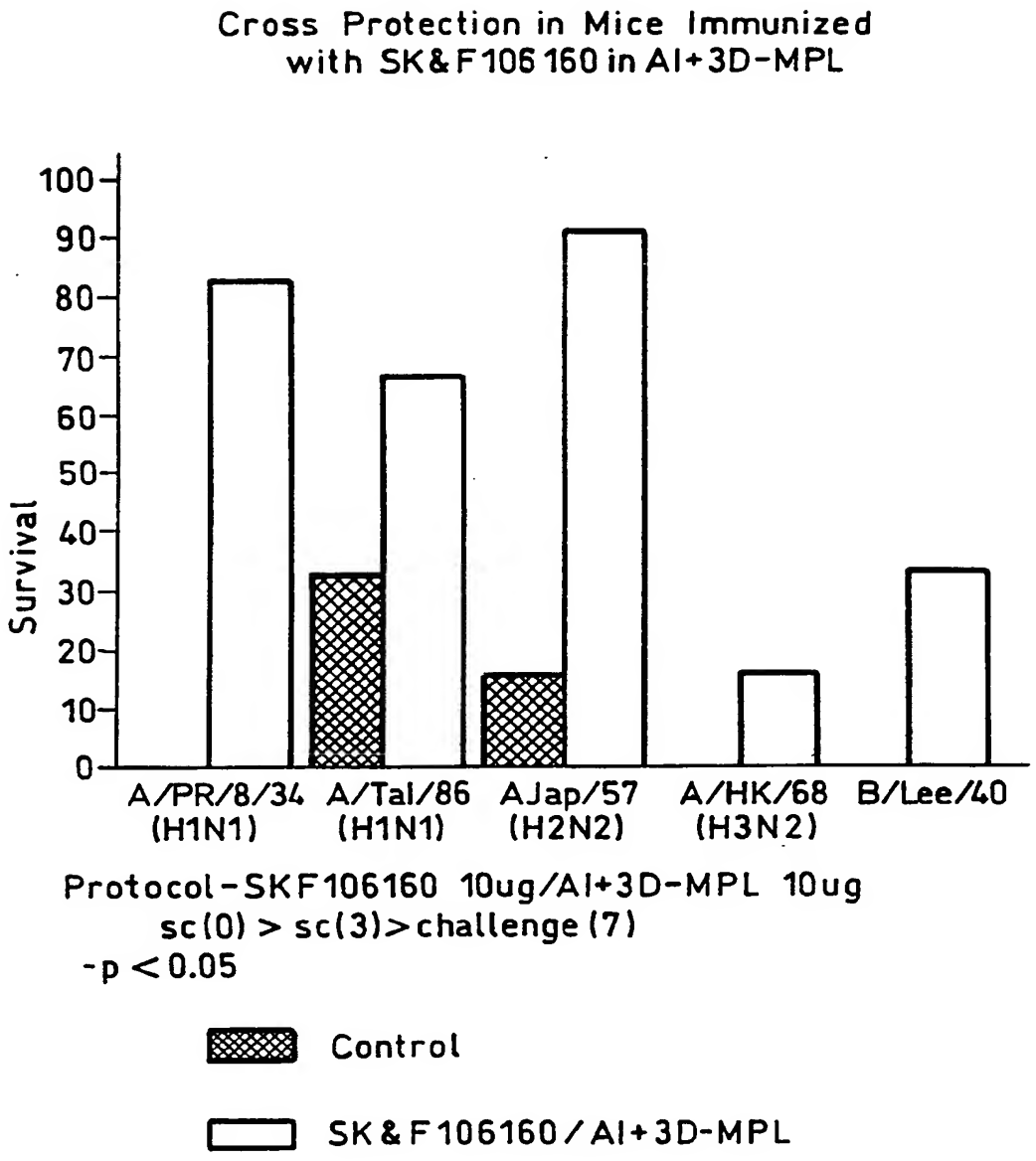


Fig. 1

SUBSTITUTE SHEET

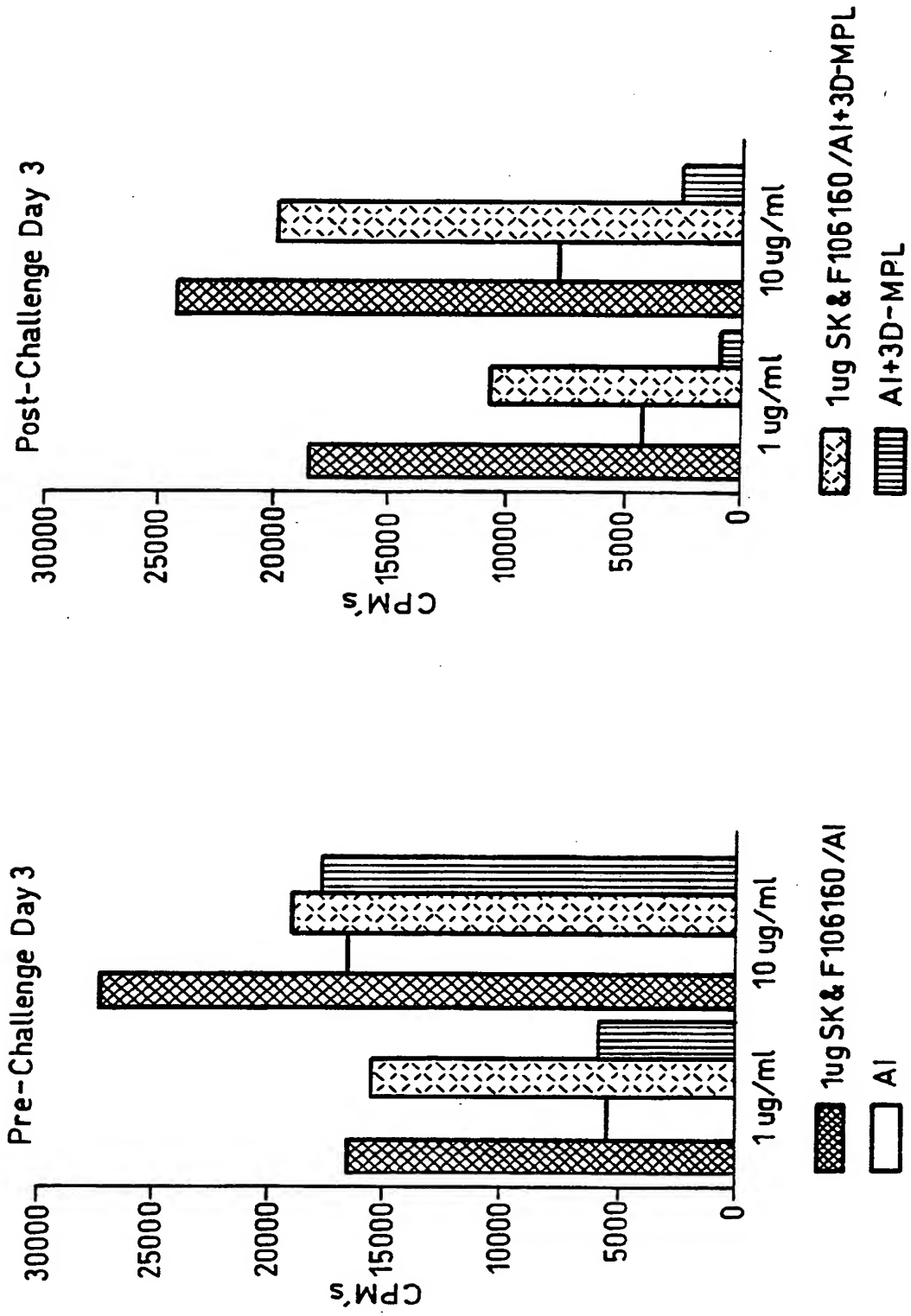


Fig.2A

Fig.2B

SUBSTITUTE SHEET

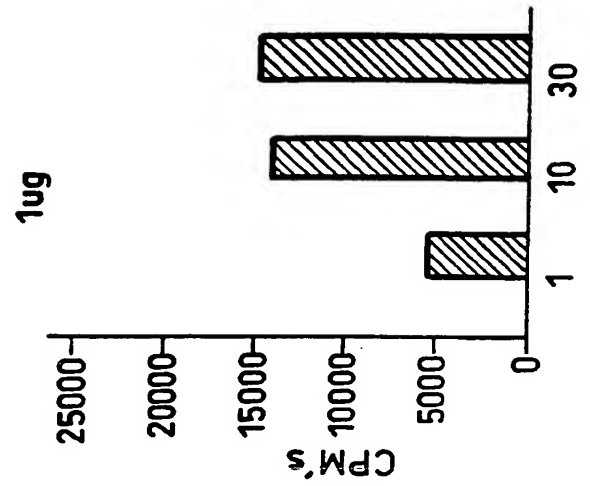


Fig.3C

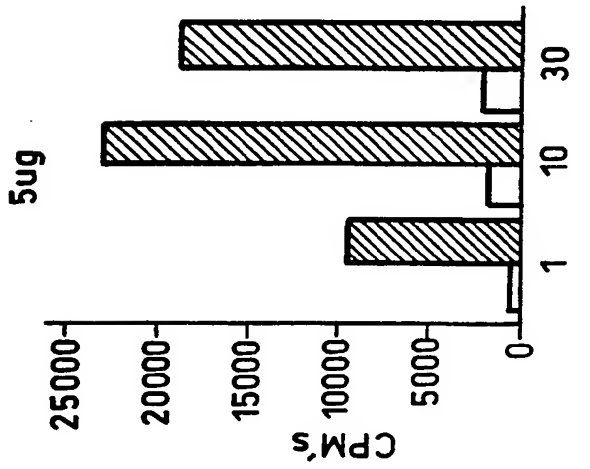


Fig.3B

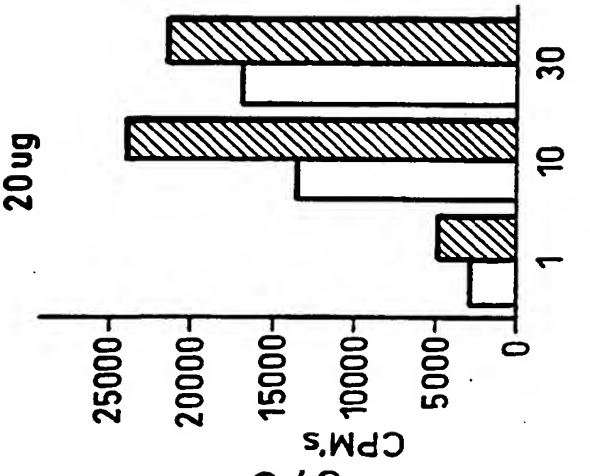


Fig.3A

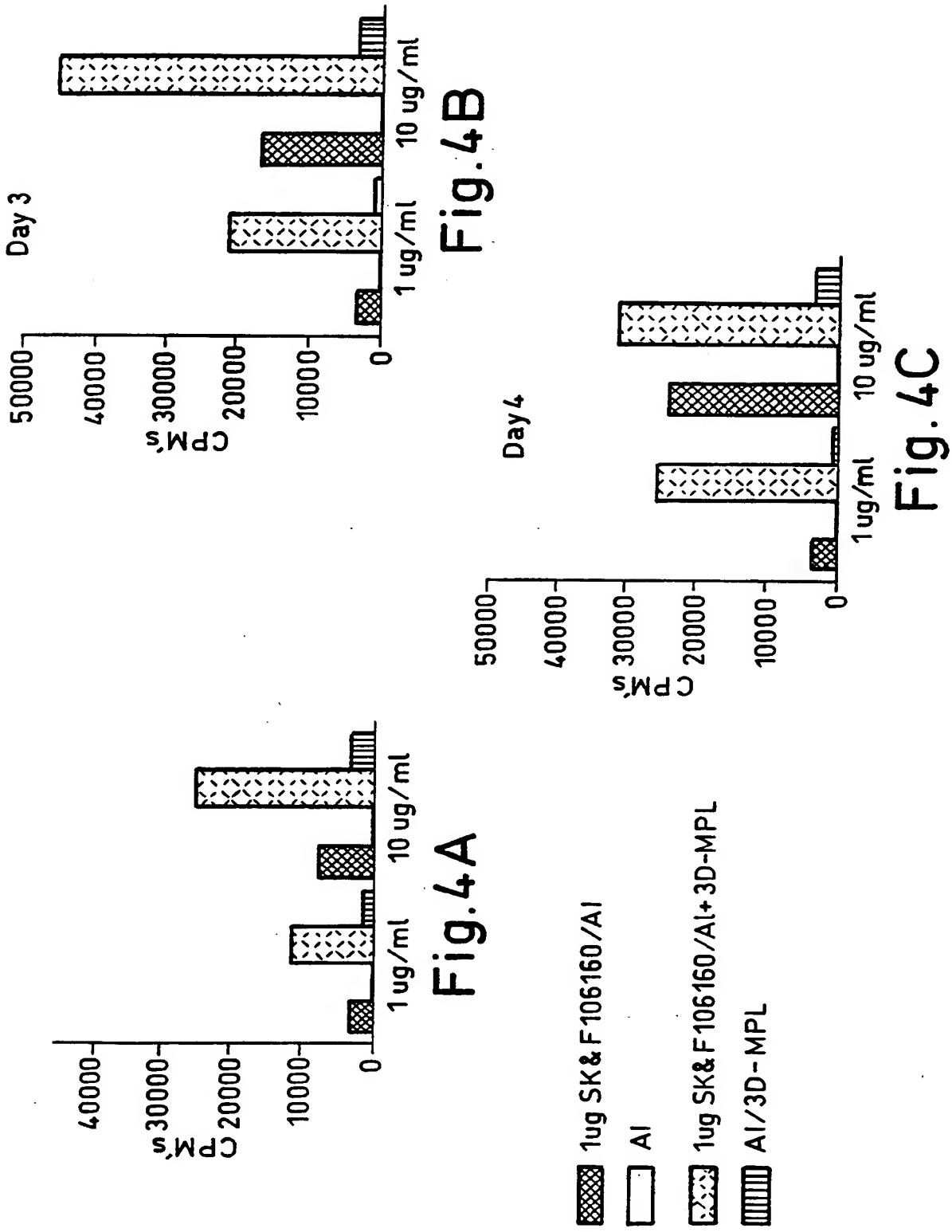


Fig.4A

Fig.4B

Fig.4C

SUBSTITUTE SHEET

Fig. 4E

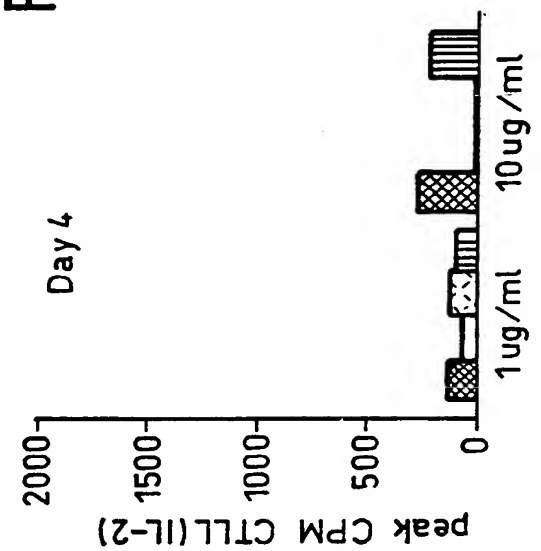


Fig. 4F

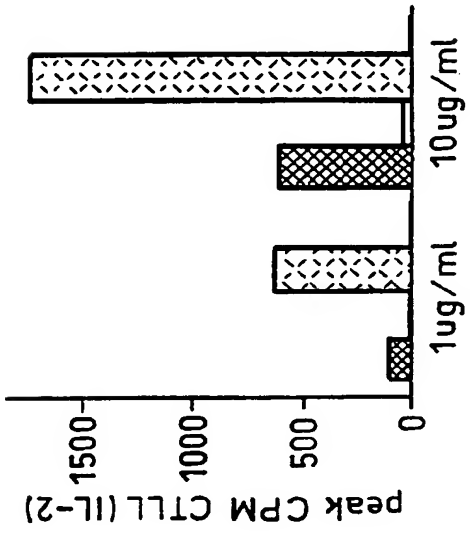
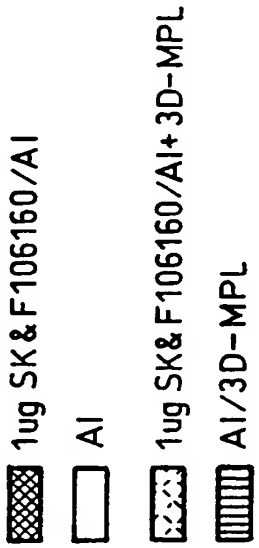


Fig. 4D



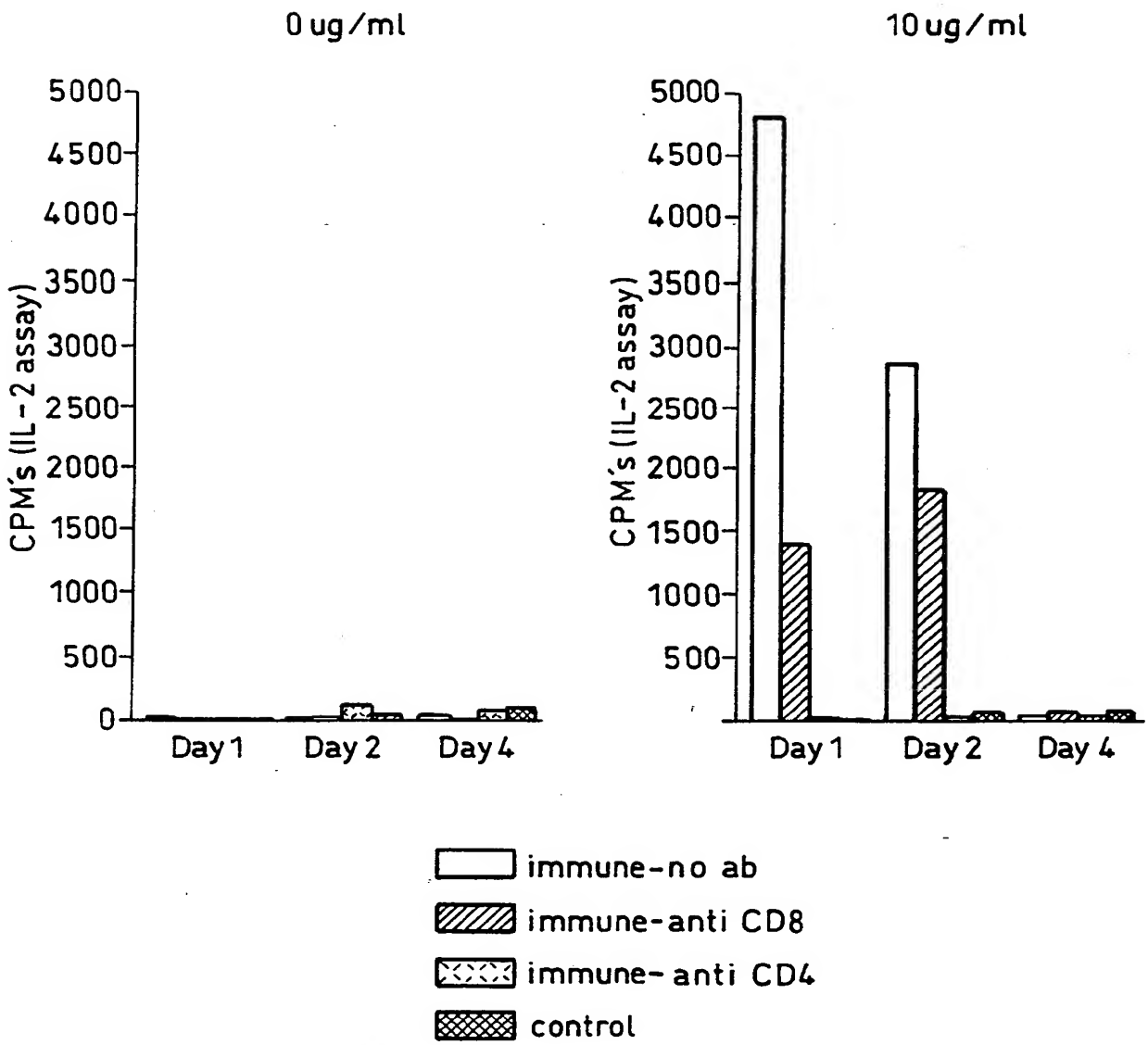


Fig. 5A

SUBSTITUTE SHEET

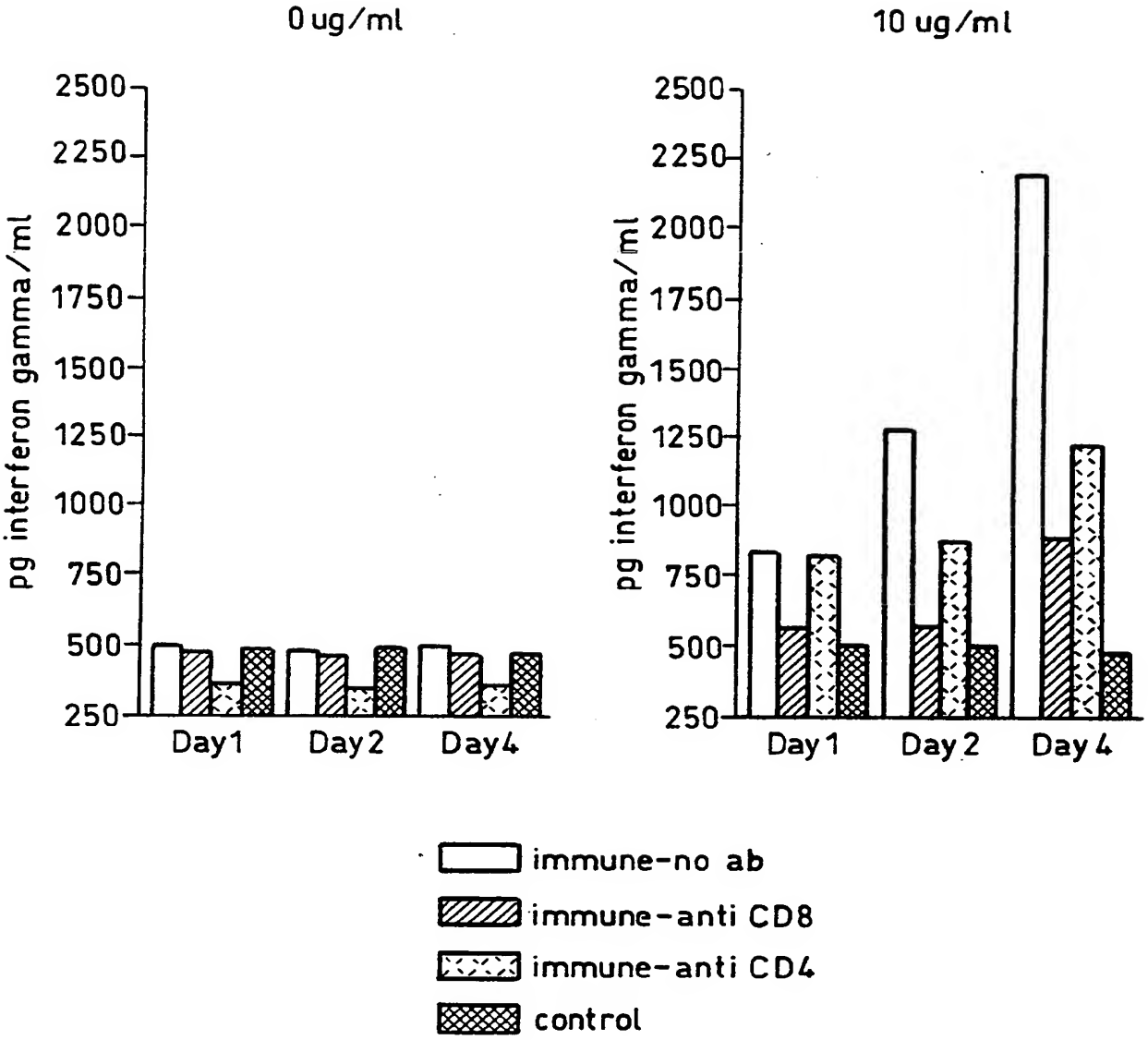


Fig. 5B

SUBSTITUTE SHEET

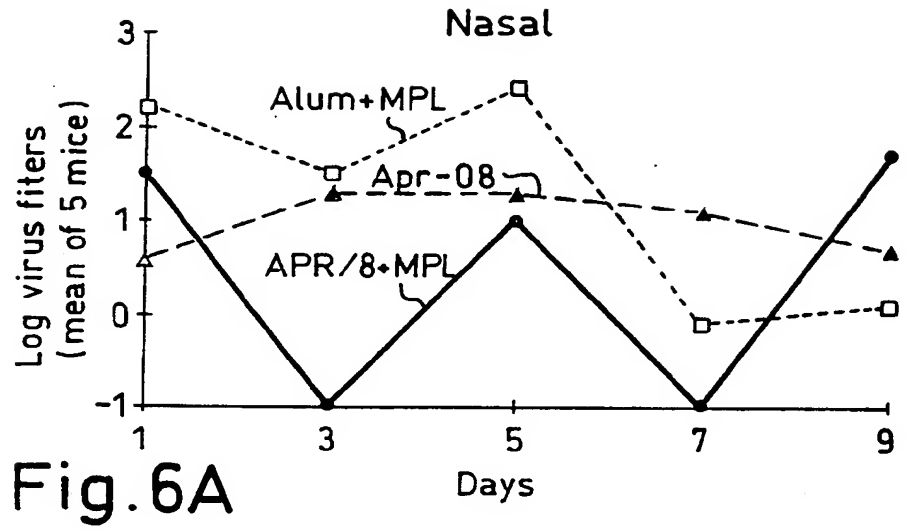


Fig.6A

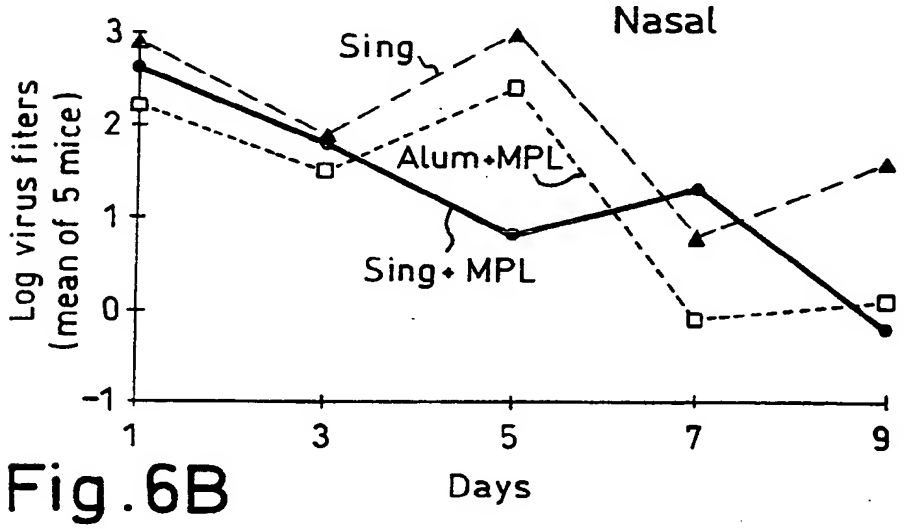


Fig.6B

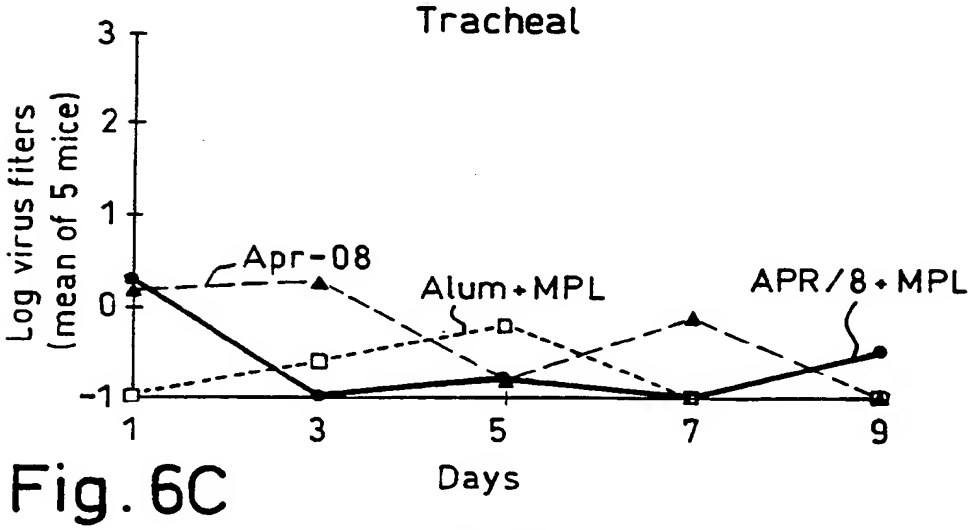


Fig.6C

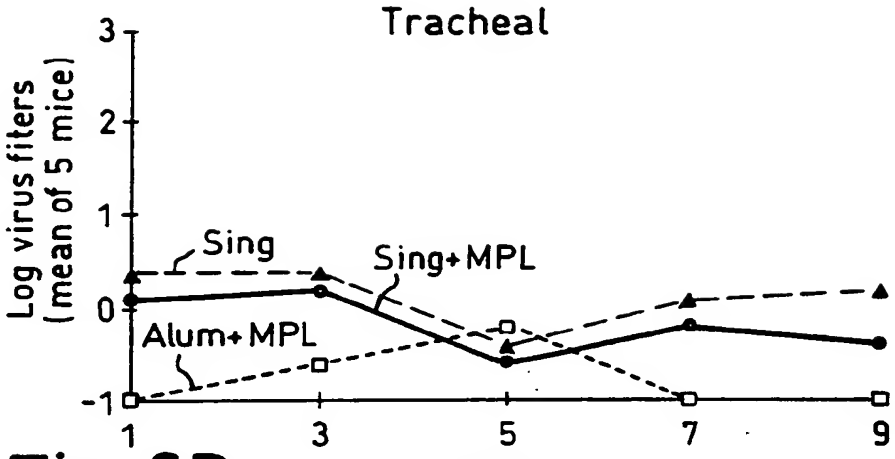


Fig.6D

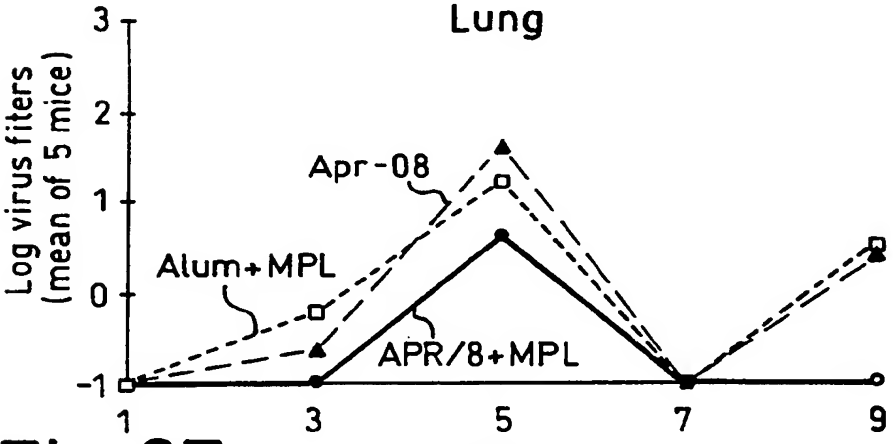


Fig.6E

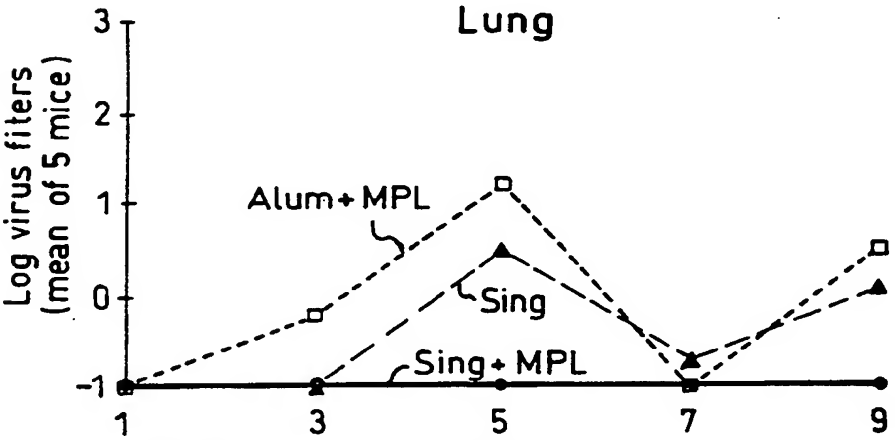


Fig.6F

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

Intr[illegible]pplication No
PCT/EP 94/00448

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K39/145 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 16231 (SMITHKLINE BEECHAM BIOLOGICALS S.A.) 1 October 1992 see page 2, line 8 - page 3, line 35 ---	1-24
Y	WO,A,92 11291 (SMITHKLINE BEECHAM BIOLOGICALS (S.A.)) 9 July 1992 see page 2, line 19 - page 9, line 18 see page 13, line 16 - page 15, line 5 ---	1-24
Y	WO,A,92 06113 (SMITHKLINE BEECHAM BIOLOGICALS (S.A.)) 16 April 1992 see page 7, line 5 - page 10, line 35 ---	1-24
Y	GB,A,2 220 211 (RIBI IMMUNOCHEM RESEARCH INC.) 4 January 1990 cited in the application see page 15, paragraph 2 - page 16, paragraph 3 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 1994	15 -06- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intr al Application No
PCT/EP 94/00448

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 366 238 (SMITHKLINE BEECHAM CORPORATION) 2 May 1990 cited in the application see claims 1-21	1-3,13, 19-24
Y	EP,A,0 366 239 (SMITHKLINE BEECHAM CORPORATION) 2 May 1990 cited in the application see page 3, line 25 - page 7, line 10	1-3, 8-13, 19-24
Y	EP,A,0 356 339 (THE LIPOSOME COMPANY,INC.) 28 February 1990 see claims 1-20	14-18
Y	EP,A,0 211 647 (ALLERGAN PHARMACEUTICALS, INC.) 25 February 1987 see claims 1-19 & US,A,5 230 899 (SMITHKLINE BEECHAM CORPORATION) cited in the application	17,18
Y	WO,A,92 13002 (PASTEUR MERIEUX SERUMS ET VACCINS) 6 August 1992 see page 1, paragraph 5 - page 3, paragraph 4; examples 1,2	4-7
P,Y	WO,A,94 00153 (SMITHKLINE BEECHAM BIOLOGICALS) 6 January 1994 see claims 1,6	1-24

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

[redacted]al application No.
PCT/EP 94/00448

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.